Role of irritation and mast cell mediators on thymic stromal lymphopoietin (TSLP) expression in the skin and its impact on severity of atopic dermatitis

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DEDICATION

I dedicate this work to my parents (Maa Smt. Sheela Devi and Babuji Shri Ramniwas Redhu) and my Uncle Shri Satbir Singh Redhu

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LIST OFABBREVIATIONS

-/-	Knockout
αh	Anti-human
αm	Anti-mouse
ANOVA	Analysis of variance
AD	Atopic dermatitis
β-Me	β-mercaptoethanol
bp	Base pair
BSA	Bovine serum albumin
C48/80	Compound 48/80
C57BL/6	C57 black 6
CASY	CASY® Cell Counter
CCL	Chemokine ligand
CD	Cluster of differentiation
ChIP	Chromatin immunoprecipitation
DNA	Deoxyribonucleic acid
cDNA	Complementary deoxyribonucleic acid
dsDNA	Double-Stranded DNA
CLA	Cutaneous lymphocyte-associated antigen
CT	Threshold cycle value
CXCL8	CXC ligand 8
DC	Dendritic cell
dDCs	Dermal dendritic cells
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme linked immunosorbent assay
FBS	Fetal Bovine Serum
Fc	Fragment crystallizable of Ig
FcεRI	Fc epsilon receptor I
Fig.	Figure

g	Acceleration of gravity
GM-CSF	Granulocyte-macrophage colony-stimulating factor
H1R	Histamine 1 receptor
H2O2	Hydrogen peroxide
H4R	Histamine 4 receptor
HCl	Hydrochloric acid
HMGB1	High mobility group box chromosomal Protein 1
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
hrs	Hours
HRP	Horseradish peroxidase
IFNγ	Interferon gamma
Ig	Immunoglobulin
ICAM-1	Intercellular adhesion molecule-1
IP	Immunopreciptation
IL-	Interleukin-
IL-7Rα	Interleukin-7 receptor alpha
IL-1Ra	Interleukin-1 receptor antagonist
i.d	Intradermal
JAK	Janus Activated Kinase
JNK	c-Jun N-terminal kinases
KCs	Keratinocytes
kDa	Kilodalton
LTα	Lymphotoxin α
LTC4	Leukotriene C4
MΦ	Macrophage
MAP	Mitogen-activated protein
MCs	Mast cells
MDM2	Murine double minute 2
MgCl2	Magnesium Chloride

mMCP6	Mouse Mast Cell Protease 6
mRNA	Messenger ribonucleic acid
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHBE	Normal Human Bronchial Epithelial
NK	Natural killer
p38	Phospho 38
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline + Tween-20
PCR	Polymerase chain reaction
PE	phycoerythrin
Pen/Strep	Penicillin and streptomycin
PGD2	Prostaglandin D2
Plcb 3	Phospholipase C-Beta 3
PMA	Phorbol Myristate Acetate
Poly I:C	Polyinosinic:polycytidylic acid
RANTES	Regulated on Activation Normal T Cell Expressed and Secreted
rh	Recombinant human
rm	Recombinant mouse
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT	Reverse transcriptase
SB	Stratum basale
SEM	Standard error of the mean
SC	Stratum corneum
SCF	Stem cell factor
SDS	Sodium dodecyl sulphate
SG	Stratum granulosum
SLS	Sodium lauryl sulphate
SS	Stratum spinosum
STAT6	Signal Transducers and Activators of Transcription 6
TAE	TRIS-Acetat-EDTA

TBS	Tris-buffered saline
TEWL	Transepidermal water loss
TGF-β	Transforming growth factor beta
Th	T-helper
TLR	Toll like receptor
TNF-α	Tumor necrosis factor-α
TNFR	Tumor necrosis factor receptor
TPA	12-o-Tetradecanoylphorbol-13- acetate
Treg	Regulatory T cell
TSLP	Thymic stromal lymphopoietin
TSLPR	Thymic stromal lymphopoietin receptor
qPCR	quantitative PCR
UTR	Untranslated region
UV	Ultraviolet
Wt	Wildtype (C57BL/6)

ABSTRACT

The skin is the first line of defense against environmental or microbial pathogens. A deviation of the skin barrier homeostasis by any kind of insult can result in an inflammatory response. The inflammatory response in turn can promote the development of an eczemaincluding atopic eczema. Thymic stromal lymphopoietin (TSLP) due to its pleiotropic nature play an important role in inflammatory disorders.

The major aim of this thesis was to better understand the underlying mechanisms of TSLP production in the context of skin irritation and mast cell (MC) mediators and their contribution in the development of atopic dermatitis (AD). The role of TSLP was studied using TSLPR-/- mice. The data show that TSLPR-/- and TNF-/-/TSLPR-/- mice were protected from AD development, by contrast TNF-/- mice exhibited severe AD. The role of exogenous triggers was studied using tape stripping mediated skin irritation mouse models. Skin irritation resulted in significant enhanced TSLP production. TSLP induction was identified to depend on interleukin (IL)-1 and protease activated receptor (PAR)-2 pathways proven by using exogenous activators or inhibitors of these pathways. Moreover, PAR-2 and IL-1 concomitantly promoted NF-κB binding to the human TSLP promoter which in turn resulted in an increased TSLP promoter activity. Additionally, the role of mast cell mediators in the context of TSLP induction was investigated. Tryptase turned out to be the trigger responsible for the enhanced TSLP response by activating the PAR-2 pathway. This finding was proven by employing *in vitro*, *ex vivo* and *in vivo* approaches. In detail PAR-2-/- and MC-/- mice were used in a compound 48/80 (C48/80) dependent MCs degranulation model. PAR-2-/- and MC-/- mice produced significantly less TSLP in comparison to control mice. Finally, these observations were confirmed in human primary keratinocytes (KCs) were tryptase and IL-1 mediated activation of the PAR-2 pathway in concert with the IL-1 pathway to elicit TSLP production.

Schlagwörter: Thymic stromal lymphopoietin, Hauterkrankungen, Atopischen Dermatitis, Mastzellen, Interleukin-1

Key Words: Thymic stromal lymphopoietin, Skin inflammation, Atopic Dermatitis, Mast cell, Interleukin-1

ZUSAMMENFASSUNG

Die Haut ist das größte Organ des Menschen und stellt die primäre Barriere gegen Umwelteinflüsse und Pathogene dar. Eine Dysbalance der Hautbarriere birgt die Gefahr einer nachfolgenden Entzündungsreaktion. Bleibt diese bestehen, können sich Hautkrankheiten wie zum Beispiel die atopische Dermatitis (AD) entwickeln. Verschiedene Zytokine wie Thymic Stromal Lymphopoietin (TSLP) werden bei entzündlichen Hauterkrankungen eine bedeutende Rolle zugeschrieben.

Hauptziel der vorliegenden Arbeit war die Aufklärung von Mechanismen, die durch Hautirritation ausgelöst oder durch Mastzellmediatoren zu einem Anstieg von TSLP als Wegbereiter einer Entzündungsreaktion in der Haut stattfinden.

Die Bedeutung von TSLP wurde zunächst anhand mehrerer Knockout-Mausstämme untersucht. Hier zeigte sich, dass TSLPR-KO und TNF-TSLPR-DKO Mäuse im Gegensatz zu TNF-KO Mäusen keine bzw. weniger Zeichen einer Entzündungsreaktion in der Haut entwickeln. Die Rolle äußerer Einflüsse auf die TSLP-Produktion wurde anhand eines Irritationsmodells ebenfalls in Mäusen untersucht. Dabei führte die Hautirritation, ausgelöst durch Abtragen der oberen Hautschichten mittels eines Tesa-Abriss, zu einem signifikanten Anstieg von TSLP in der Haut?. Mit Hilfe von Agonisten und Inhibitoren konnte gezeigt werden, dass dieser Irritations-vermittelte TSLP-Anstieg über Interleukin-1 (IL-1) und Protease Activated Receptor 2 (PAR-2) vermittelt wird. In diesem Zusammenhang wurde auch gezeigt, dass die Aktivierung von IL-1- sowie PAR-2-abhängigen Signalwegen zu einer gesteigerten Aktivität des TSLP-Promotors führte.

Die Untersuchung der Wirkung verschiedener Mastzellmediatoren auf die TSLP-Expression ergab, dass Tryptase, über die Aktivierung von PAR-2, der wichtigste Mediator für den Anstieg von TSLP nach der Degranulation von Mastzellen ist. Dieses Ergebnis wurde mittels verschiedener *in vitro*, *in vivo* und *ex vivo* Experimentalansätze belegt. So konnte in einem c48/80-abhängigen Degranulationsmodell in Mäusen gezeigt werden, dss PAR-2- sowie Mastzell-KO Mäuse im Vergleich zu Wildtypen nach Injektion von c48/80 signifikant weniger TSLP exprimierten. Abschließend konnte das Zusammenspiel von PAR-2- und IL-1-vermittelten Signalwegen in Bezug auf TSLP in humanen Keratinozyten bestätigt werden.

Schlagwörter: Thymic stromal lymphopoietin, Hauterkrankungen, Atopischen Dermatitis, Mastzellen, Interleukin-1

Key Words: Thymic stromal lymphopoietin, Skin inflammation, Atopic Dermatitis, Mast cell, Interleukin-1

1 INTRODUCTION

1.1 SKIN STRUCTURE

The skin acts as a protective barrier and protects the body from a wide range of potential harmful pathogens by separating inner and outer environment.[1] The skin is built of three layers, the outer epidermis, the dermis and the subcutis respectively.[2] The epidermis is of utmost relevance for the barrier integrity of the skin and confers the body with physical, chemical or biochemical protections. The epidermis consists of various layers of keratinocytes which go through the process of differentiation. These are the innermost stratum basale, the stratum spinosum, the stratum granulosum and upper most the stratum corneum.[1,3,4] The stratum basale layer contains basal stem cells, which are capable of proliferation to generate keratinocytes and can expand the cell numbers.[5] The stratum spinosum is defined by evident desmosomes, which help the appearance of spindle shaped cells. Early differentiation marker cytokeratin 10 is expressed by the cells of this layer. Differentiation of cells can be seen from bottom to top by the visibility of *involucrin*, an intermediate differentiation marker, in upper cell lyers but not in the lower spinous cell layers. The center part of the skin mainly consists of flat anucleated corneocytes. These anucleated cells contain keratin filaments which symbolize differentiated keratinocytes of the outer stratum granulosum layer.[1,3,4] The stratum granulosum is composed of 3-5 layers of cells and is characterized by the presence of lamellar bodies as well as keratohyalin granules. The two late differentiation markers *filaggrin* and *loricrin* are expressed and processed by these cell layers.[6] The stratum corneum predominantly forms the primary skin barrier against cutaneous infiltration of chemicals, microbes and mechanical injuries.[1,7] In the stratum corneum cells are held together by lipid bilayers, which form a rigid and insoluble structure known as cornified envelope. The stratum corneum is also involved in various active processes like regulation of water loss to outer environment from the skin, known as transepidermal water loss (TEWL).[1,7]

The thickest part of the skin is the dermis containing sweat glands, sebaceous glands and hair follicles among other structures.[8] The dermis also harbors a complex network of blood vessels and capillaries in addition to connective tissue. Thermoregulation of body is regulated by the dilation or contraction of these blood vessels.[8] Collagen and elastin, which are present in the dermis provide plasticity.[9]

1.2 EPIDERMAL BARRIER AND IT'S DISRUPTION IN ATOPIC DISEASES

The skin is metabolically active and several physiological phenomena help to maintain the skin barrier intact. Protection of the inner body from microorganisms in addition to physical, chemical, thermal and mechanical danger is the fundamental function of the skin (Fig.1).[10] Several factors are required to sustain the skin barrier function. These include prevention of excessive water loss, renewal of skin cells, cell to cell communication and interaction with the immune system. Upon epidermal barrier disruption, the initiatory reaction to cellular damage of the epidermal cells is a stimulatory alert to substitute the damaged cells and to maintain the homeostasis in the skin.[11] Immune cells like epidermal Langerhans cells (LCs), dendritic cells (DCs) which are also known as skin-resident immune cells, are major players in homeostatic reestablishment.[10] In response to skin injury, keratinocytes (KCs) produce pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), Interleukin (IL)-1α, β, IL-6 and IL-18. These cytokines activate dermal DCs in the presence or absence of antigens. When KCs get activated due to stress signaling, they participate in further activation of dermal DCs by secreting interferon-α (IFN-α) (Fig. 1). Upon activation DCs boost skin-resident CD4+ or CD8+ T cell proliferation (Fig. 1). Activated T cells amplify the inflammatory response in skin by producing chemokines and cytokines which in turn act on epithelial and mesenchymal cells such as KCs and fibroblasts (Fig. 1).[10]

1.2.1 Skin irritants - physical and chemical

The skin is susceptible to exposure by different irritants which can result in detrimental effects on barrier function and a subsequent damage of the epithelial cells.[12] Several studies have been conducted to better understand the underlying mechanisms of acute and chronic irritation[12]. However, to study the pathogenesis of irritation at a cellular level in humans is difficult due to ethical reasons. Therefore mouse models were employed to study the physico-chemical phenomena behind these reactions. Many studies have been reported using different chemical and physical irritants such as sodium dodecyl sulphate (SDS), croton oil, acetone, tape stripping.[13] Perturbed skin barrier assessment is analyzed by measuring TEWL, electrical capacitance (stratum corenum hydration), percutaneous drug transport and skin color reflectance (erythema).[13,14] A strong inflammatory response has been observed by Willis *et al*. (1993) on exposure of murine skin to 5% SDS for 48 hours (hrs).[15] It has been shown that SDS at higher concentrations leads to down regulation of HLA-DR expression in

the LCs.[16] Tape stripping mediated barrier disruption is another common method used to study irritation, with less cytopathic effect on epithelial cells. Adhesive tape strip is commonly used to remove the layers of stratum corneum.[17] Upon stratum corneum disruption, TEWL increases and leads to the production of different pro-inflammatory mediator.[17,18]

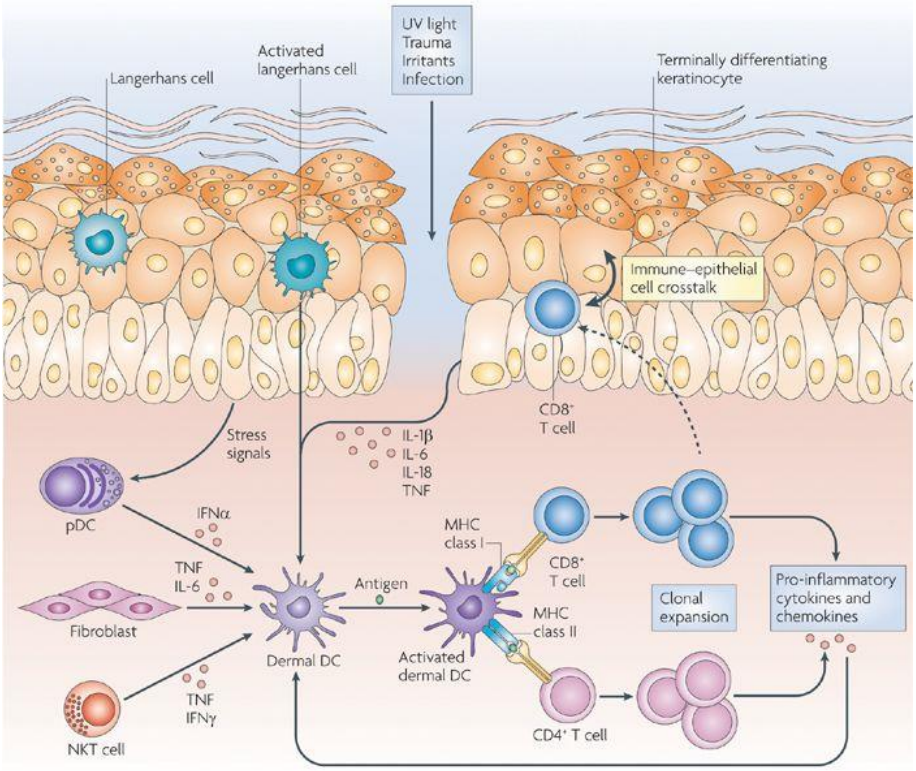


Figure 1: Skin barrier disruption results in inflammatory response.

Mechanical injury and exposure to infectious agents or irritant results in skin barrier disruption and initiates inflammatory response. Keratinocytes upon barrier damage start producing pro-inflammatory cytokines such as IL-1β, IL-6, IL-18 and TNF-α which lead to activation of the dendritic cells followed by activation of mast cells and T cells. (reprinted from Skin immune sentinels in health and disease. Frank O. Nestle, Paola Di Meglio, Jian-Zhong Qin and Brian J. Nickoloff, Nat Rev Immunol. Oct 2009; 9(10): 679–691, copyright 2009, with permission from Springer nature).

In alignment with the murine findings, similar pro-inflammatory immune responses was reported in human KCs by different chemical irritants such as croton oil, SLS and phenol[19,20].

1.2.2 Atopic dermatitis (AD)

AD is a heterogeneous chronic-relapsing skin disease. It is characterized by the presence of erythema, eczema, edema, excoriation, xeriosis, pruritus and a typical localization pattern[21]. Generally, commencement of AD starts during childhood.[21,22] Epidemiological studies worldwide indicate an increase in the frequency of AD development in the last decades affecting nearly 10-20% and 1-3% of the children and adult populations, respectively.[22-24]

Genetic and environmental factors play an important role in the development of AD but the underlying molecular mechanisms of AD are still not clear.[25,26] The contribution of immune dysregulation and genetic factors as a consequence of which AD development occurs are still not clearly defined.[22,26,27] Major events and different cells types involved in the development of AD are shown in figure 2.[28] Genetically, a null mutation in the *filaggrin* gene encoding for the important barrier protein *filaggrin*, reported to be present in 20% of AD patients.[24,25,29] Skin barrier is formed of *filaggrin* and several other proteins which may also play important role in the AD development. Furthermore AD development can be surpassed even though in the presence of *filaggrin* mutations indicating disruption of the skin barrier not sufficient for the disease development.[30,31] Other T-cell types like T-reg, Th17, Th9 and Th22 have been reported to play a roles in AD pathogenesis but their explicit role in the progression of AD is not well defined (Fig. 2).[32,33]

Keratinocytes are key components during the initiation of the disease by activating DCs leading to Th2 polarization, through secretion of high amounts of TSLP in atopic skin (Fig. 2).[21] Although T cells were previously considered to be critical for pathogenesis of AD and are redundant under particular conditions. These T cells can be substituted by innate immune cells including mast cells (MCs), eosinophils and macrophages.[34-36] Thus, based on heterogeneity of cells and signaling cascades involved in AD which ultimately converge on barrier disruption, it is conceivable that perturbations in skin cells and immune deviations can participate in AD independently and trigger its development.[37]

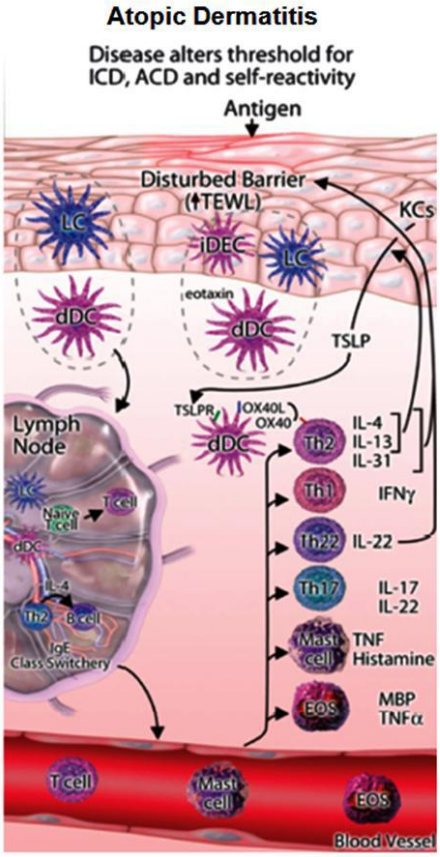


Figure 2: Immune mechanism in the pathogenesis of AD.

In patients with AD, a disturbed epidermal barrier leads to increased permeation of antigens, which encounter Langerhans cells (LCs), inflammatory dendritic epidermal cells *(iDECs)*, and dermal dendritic cells (dDCs), activating T_H2 T-cells to produce IL-4 and IL-13. DCs then travel to lymph nodes, where they activate effector T-cells and induce IgE class-switching. IL-4 and IL-13 stimulate KCs to produce TSLP. TSLP activates OX40 ligand–expressing dDCs to induce inflammatory T_H2 T-cells. Cytokines and chemokines, such as IL-4, IL-5, IL-13, eotaxins, CCL17, CCL18, and CCL22, produced by T_H2 T-cells and DCs stimulate skin infiltration by DCs, mast cells, and eosinophils (EOS). T_H2 and T_H22 T-cells predominate in patients with AD, but T_H1 and T_H17 T-cells also contribute to its pathogenesis. The T_H2 and T_H22 cytokines (IL-4/IL-13 and IL-22, respectively) were shown to inhibit terminal differentiation and contribute to the barrier defect in patients with AD. Thus both the barrier defects and immune activation alter the threshold and self-reactivity in patients with AD (reprinted from *Journal of Allergy and Clinical Immunology*, Vol 131, Issue 2, JK Gittler, JG Krueger, and E Guttman-Yassky, 300–313, copyright 2013 with permission from Elsevier).

1.3 KERATINOCYTES

KCs are epithelial cells which conserve the physical and biochemical integrity of skin.[38,39] During differentiation to form skin barrier, KCs undergo complex morphological and cytostructural changes with the expression of different differentiation-dependent structural proteins such as *involucrin, filaggrin, claudin* etc. in the spinous and granular layers.[38] KCs play crucial role in the cellular communication, pathogenesis of diseases and in maintaining the immune response.[31,40,41] Deviation from skin homeostasis or barrier disruption act as activation signal for KCs in response to which they start producing different pro-inflammatory cytokines such as TNF-α, IL-1α, TSLP to facilitate inflammation.[10]

1.3.1 Role of keratinocytes in skin irritation

As pointed out above, for maintaining skin homeostasis KCs are the crucial cells. By undergoing differentiation, they form rigid structure and consecutively enter into cell cycle arrest phase.[42] IL-1α produced by KCs acts as primary alarm signal upon skin irritation or other skin disruption in the inflammatory pathway.[43] Numerous, studies have demonstrated the ability of different irritants to induce IL-1α production in KCs, which further boost the production of other pro-inflammatory cytokines such as IL-1β, IL-6, IL,18, TNF-α by dermal and epidermal cells.[42,44-47] Physical or chemical skin irritation leads to activation of proteases which cleave the pro-IL-1β into biologically active IL-1β which along with IL-1α support the activation of DCs and T cells.[48]

1.3.2 Role of keratinocytes in AD

Barrier deficiency in AD development and progression is primarily caused by KCs.[49] KCs produce peculiar set of cytokines and chemokine's such as high levels of chemokine ligand (CCL)5 (RANTES), thymic stromal lymphopoietin (TSLP) upon stimulation with IL-1and TNF-α to promote AD environment.[20,50] It has been reported that KCs derived from AD patients produce high amount of granulocytes-macrophage colony-stimulation factor (GMC-SF) in addition to TNF-α.[51] Recent studies, demonstrating the involvement of KCs derived cytokines such as TSLP on the inflammatory response provide a deeper insight for the role of KCs not only in barrier formation, but also as collaborative cells along with DCs activation to primer T cells to induce production of IL-4 and IL-13.[50,52] TSLP mediated activation of DCs

results in production of chemokines such as CCL17, macrophage derived CCL22, which further foster infiltration of Th2 cells in the lesional AD skin.[28] It has been shown that upon activation KCs produce IL-25 and IL-33 which act on mast cells (MCs), DCs and LCs.[31,34]

1.4 MAST CELLS

MCs are bone marrow derived cells which migrate to tissue through blood for maturation under the influence of stem cell factor (SCF). They can be identified by staining for tryptase. Skin MCs are belong to the connective tissue type and contain both chymase and tryptase (MC_{TC}) while the mucosal MCs contains only tryptase and no chymase (MC_T).[53-55] MCs are deemed to be among the first cells to respond to an allergen/injury and are considered as the instigating players in the IgE-mediated immediate type hypersensitivity. MCs degranulation can be mediated by human G protein coupled receptor MAS Related GPR Family Member X2 (MRGPRx2) activation by certain drugs, substance P or compound 48/80 (C48/80) in addition to classical FcεRI crosslinking by polyvalent allergen/agent through binding to IgE present on FcεRI.[56, 57,58]

1.4.1 Role of mast cells in AD

Profound degranulation of MCs along with their recruitment is observed in the lesions of AD skin.[54,59] MCs degranulation has been shown to correlate with the severity of AD.[60] MCs can regulate the recruitment as well as functions of cells participating in the skin inflammation through the production of cytokines, chemokines and growth factors (Fig. 3).[61] For instance, by virtue of IL-4, IL-13 and TNF-α they induce cell adhesion molecule on endothelium, which can contribute in the recruitment of leukocytes.[62-64] They can also control the differentiation of naïve T cells to Th1 or Th2 subtypes and increase the activation of T cells.[65,66] MCs can also modulate primary B cell development and induce IgE synthesis in B cells.[66-68]

MCs can interact with KCs, DCs and LCs by their mediators. By secreting tryptase (endogenous PAR-2 agonist) and histamine, MCs can stimulate KCs to express pro-inflammatory chemokines and cytokines, growth factors and adhesion proteins.[69,70] They can induce integrin on the LCs by TNF-α and promote their migration to lymph nodes.[71,72,73] Moreover, MCs affect DCs polarization to Th1/Th2 through mediators and

cytokines.[74] Additionally, MCs can act like professional antigen-presenting cell by directly presenting antigen to T cells.[75-79]

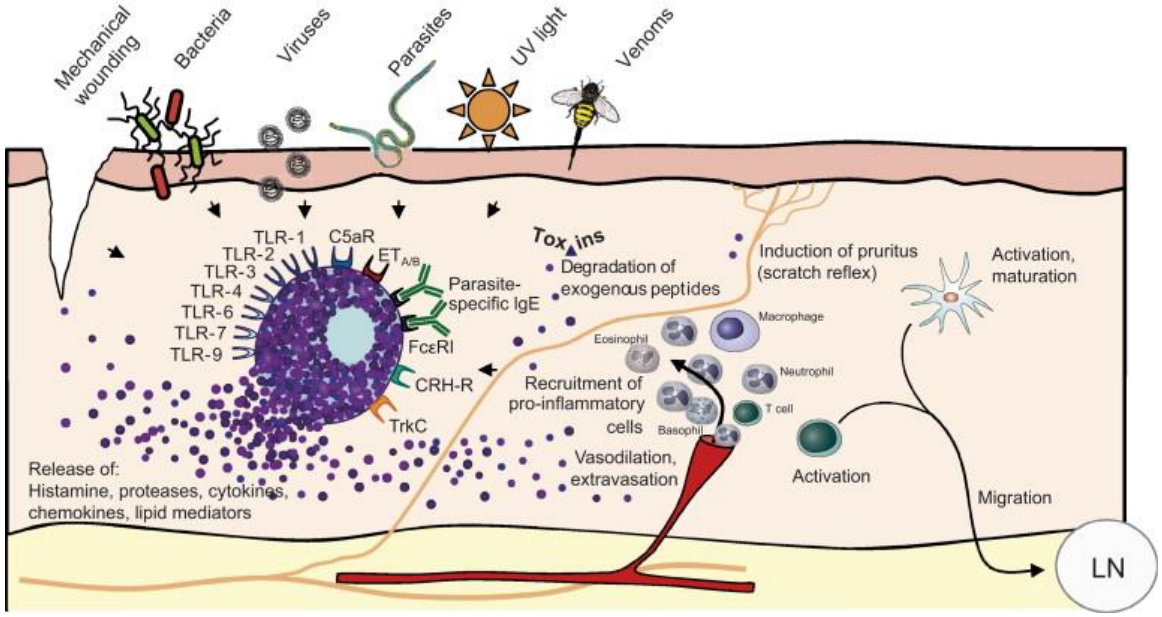


Figure 3. Skin mast cells sense and react to various danger signals.
TLR, toll-like receptor; ET, endothelin; CRH, corticotropin releasing hormone; LN, lymph node (reprinted from Metz et al. 2007, Immunobiology 213 (2008) 251–260, copyright 2007 Elsevier GmbH., with permission from Elsevier)

1.5 PROTEASE ACTIVATED RECEPTOR (PAR)-2

The KCs sense the extracellular proteolytic activity by a unique class of G-protein-coupled receptors (GPCRs), called PARs. Four PAR receptors have been reported with special cellular functions. Proteolytic cleavage of N-terminal end results in activations of PARs. Thus, a tethered ligand sequence (TLS) is exposed that binds to the second extracellular loop of the same receptor and sequentially causes receptor activation, signaling as well as receptor internalization. Contrary to other ligand binding GPCRs, such as activation and internalization of PARs leads to endosomal trafficking, ubiquitination and degradation of the receptors in lysosomes. Thereby, reconstitution of the cells surface with PARs relies on the de novo synthesis or transport of stored receptors within intracellular vesicles.[80-83] Several reports suggest that activation of PAR-2 plays a crucial role in inflammation, pain and

allergic responses.[84-88] PAR-2 activation is mediated by a broad range of endogenous proteases including serine proteases (e.g. KLK5, KLK14, tryptase) and exogenous proteases such as house dust mite (HDM) antigen Der p1.[89-92] PAR-2 activation has been shown to induce itch, either directly upon activation of receptor on sensory nerve fibers in the skin, or indirectly by activating KCs or other immune cells (e.g. MCs), and consequently elicit a cascade resulting in release of pruritogens which in turn activate the sensory nerve fibers innervating the skin.[93-96]

1.5.1 Role of PAR-2 in AD

Severe skin barrier disruption leads to excessive dehydration, chronic skin inflammation, itch and enhanced risk of skin infections. Patients with AD, netherton syndrome (NS) or ichthyosis, suffer from extensive itch and ultimately develop erythematous scaly skin as a result of faulty skin barrier.[36,97,98]

Along with kallikreins (KLKs) and endogenous protease inhibitors, PAR-2 as well as its activating proteases are important regulators of KC differentiation and skin barrier homeostasis. Interestingly, hyperkeratosis in different inflammatory skin diseases (e.g. AD or NS) is coincided by enhanced expression of PAR-2 and PAR-2 activating proteases.[36,97,99]

1.6 THYMIC STROMAL LYMPHOPOIETIN (TSLP)

TSLP is an IL-7 like cytokine and has been first identified in the mouse thymic stromal cell culture supernatants. TSLP promote the differentiation and growth of B cells in addition to proliferation of T cells.[100,101] Several studies have reported that high affinity binding of TSLP needs concomitant binding to the TSLP receptor (TSLPR) and α-chain of IL-7 receptor.[102,103] Primarily TSLP is expressed by epithelia cells of skin, gut, thymus and tonsils along with stromal cells.[104-106] TSLP leads to differentiation of Treg cells by instructing the thymic DCs.[107] Intriguingly, human TSLP does not exert the same effects as the murine TSLP, despite that it activate immature CD11c+ myeloid DCs.[106,108] Thereby, human DCs can induce naïve CD4+ T cell proliferation and triggers the production of IL-4, IL-5, IL-13 as well as TNF-α (fig. 7). On the other hand, TSLP activated DCs inhibits the production of anti-inflammatory cytokines IL-10 and IFN-γ.[108] As a broad range of cells are influenced by TSLP, it has been implied to play a major role in numerous ailments like cancer, infections and inflammatory bowel diseases.[109-111] However, TSLP being primarily an epithelial cytokines, has been expected to play a vital role in allergic diseases such as asthma and AD.[112] In line with the expected role, TSLP has been found upregulated in mouse models of

allergic asthma as well as AD and found responsible for defective airway inflammation and skin inflammation.[113-115]

1.6.1 Role of TSLP in AD

Skin barrier integrity is compromised upon acute injury or perturbation to the stratum corneum leading to induction of positive and negative alarm signals resulting in triggering of both homeostatic and inflammatory reactions in the skin.[18,116] The damaged skin barrier further fuels the production of particular cytokines to promote skin inflammation.[99,117,118] TSLP is one of the cytokine, produced by KCs on skin barrier damage or stimulation with inflammatory cytokines (fig. 4).[52] Although, the importance of TSLP in allergic inflammations is firmly established, the mechanisms behind the induction of TSLP production are not well known.[35,119,120] Primary human KCs and skin explants have been shown to produce enhanced TSLP in response to viral, bacterial or inflammatory stimuli or upon physical/chemical perturbation to skin barrier.[20,121,122] The role of TSLP in the manifestation of AD was not clear until it was reported that TSLP overexpression in the murine skin results in development of spontaneous dermatitis, the hallmark of human AD.[34,106] Since epithelial cells are the primary source of TSLP, this further indicate KCs are the trigger factor in the AD development (Fig. 4).[123] Subsequently, several studies verified TSLP as a primary initiator of AD.[35,36,124] Development of AD like skin lesions have been observed upon direct administration of TSLP in skin. TSLP promotes the proliferation and differentiation of Th2 cells and consequently expression of TNF-α, IL-4, IL-5 and IL-13.[106] Furthermore, it was observed that expression of TSLP in KCs from AD patients is highly increased. Likewise, TSLP is associated with migration and activation of dermal DCs.[125] Thus, TSLP was considered as one of the key factors in triggering AD development. Mice overexpressing KCs specific TSLP, but lacking T cells developed skin inflammation, suggesting that for the disease progression T cells are not obligatory.[34] In agreement with the previous study, other studies by using different AD models demonstrated that TSLP is essential for AD development.[115,126]

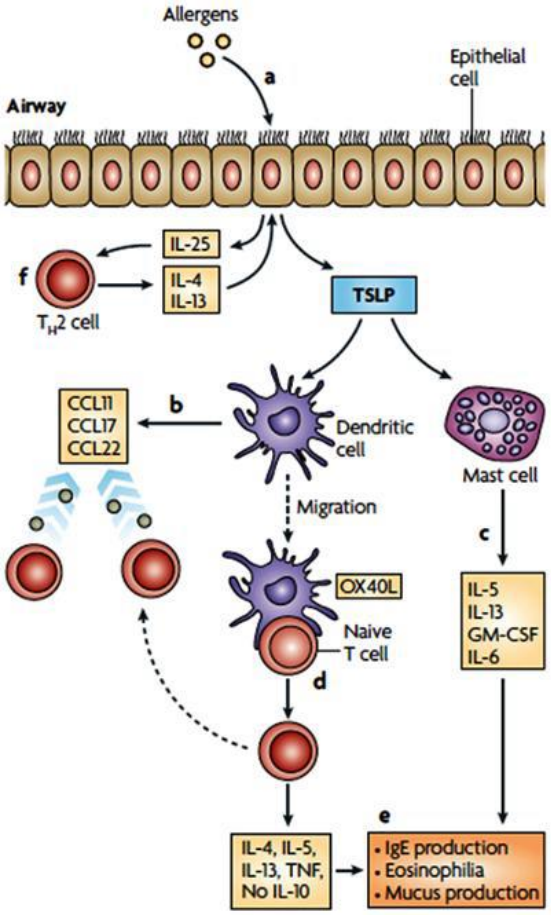


Figure 4: Induction of TSLP production by keratinocytes.

Disruption of skin barrier, allergen or cytokines derived from Th2 immune response triggers the production of TSLP by epithelial cells. Further, TSLP mediated activated DCs promote recruitment of T cells which further amplifies the production of pro-inflammatory cytokines and chemokines such as TNF-α, IL-4, IL-5. Mast cells also start producing other cytokines like IL-5, IL-13, upon activation by TSLP (reprinted from Hamida Hammad *et al.* 2008: DCs and epithelial cells: linking innate and adaptive immunity in asthma: *Nature Reviews Immunology* 8, 193-204 (March 2008), Copyright © 2008, with permission from Nature Publishing Group (2014).

1.7 OBJECTIVES

Over the years, TSLP has been well established as critical pro-inflammatory cytokines with implications in inflammatory disorders. TSLP promotes pro-allergic Th2-type inflammatory responses through activating leukocytes. However, the mechanisms of TSLP regulation in skin irritation, contribution of endogenous MCs mediators in TSLP production and its role in AD development is not clear. In this thesis, mechanisms underlying the TSLP production by skin irritation, MC mediators and its contribution in the AD development was investigated. In this thesis the following questions were tackled.

1. Are the TSLPR-/- mice protected from AD development under the TNF deficiency?
2. Is skin barrier disruption responsible for elicited TSLP production and what are the signaling cascades involved? Do the responsible pathways operate cooperatively or independently and at what levels?
3. Are the mast cells plays a role in TSLP production by the keratinocytes? And if so what are the mediators and mechanisms behind MCs and KCs cross-talk in the production of TSLP?

Answer to these questions will help in better understanding of the mechanisms underlying inflammatory processes in the AD development and may help in designing more irresolute clinical management by exclusive aiming of pathways involved individually or collectively depending on the situation.

2 MATERIALS AND METHODS

2.1 MATERIALS

Detailed list of used reagents, antibodies, solutions, labwares and softwere are given below:

Details about antibodies, instruments, chemicals, buffers, solutions, reagents, labwares and software used are listed below:

Table 1. List of reagents

Reagents	Supplier	Catalog Number
Agarose	Biozym	840004
Albumin from chicken egg white, Grade V	Sigma-Aldrich	A5503-10G
Antibody diluent (Dako REALTM)	DAKO Diagnostika	S0809
Aqua	Braun	2351744
Avidin/Biotin Blocking Kit	Vector Laboratories, Inc.	SP-2001
Beta mercapto ethanol	Sigma-Aldrich	M6250
Bovine serum albumin (BSA)	PAA	K45-001
Chromatrap enzymatic shearing kit	Chromatrap	500165
Compound 48/80	Sigma-Aldrich	C2313
DermaLife K Medium Complete Kit	Lifeline Cell Technology	LL-0007
Desloratadin	Sigma-Aldrich	D1069
Dispase	BD Biosciences	354235
Desoxyribonucleic acid (DNA) Molecuar Weight	Roche	11721925001

XIII – 50 base pair (bp) ladder		
DNA Molecular Weight XIV – 100 bp ladder	Roche	11721933001
Dulbecco´s phosphate-buffered saline (PBS)	GE Healthcare	H15-002
Ethanol	J.T. Baker	8025
Ethidium Bromide Solution	Invitrogen	15585-011
Fetal Bovine Serum (FBS)	PAA	NC9862466
High sensitivity ChIP kit	Abcam	Ab185913
Histamine	Sigma-Alrich	H7125
Human TSLP Duo Set	R&D Systems®	DY1398
Isoflurane (Forane)	Abott	
IMDM medium	PAA	E-15-819
JNJ 10191584 maleate	Sigma-Aldrich	J3830
LightCycler® FastStart DNA Master SYBR Green I	Roche	12239264001
Mouse mast cell protease(mMCP)-6	R&D Systems®	3736-SE
Mouse TSLP Duo Set	R&D Systems®	DY555
Nucleo Spin® RNA II	Macherey-Nagel, Düren	740955.250
Penicillin/Streptomycin	Biochrom	A 2212
Phorbol-12-myristate-	Sigma-Aldrich	P8139

13-acetate (PMA)		
Proteinase K	Macherey-Nagel	740506
Reverse Transcription Kit	Applied Biosystems	4368814
SLIGRL-NH_2	Tocris	1468
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich	151-21-3
Streptavidin-horseradish peroxidase (HRP)	R&D Systems®	DY998
Sulfuric acid, H2SO4	Merck	1090734000
TAE buffer (50x)	Genaxxon	M3087.1000
Trypsin / EDTA Solution	Gibco® BD	R-001-100
Tryptase	Sigma-Aldrich	650366-M
Trypsin inhibitor from *Glycine max* (soybean)	Sigma-Aldrich	T9003
Tween 20	Sigma-Aldrich	P1379-500ML
Xylol	Roth	9713.3
2-Furoyl-LIGRLO-amide	Tocris	3015

Table 2. List of antibodies

Antibody	Supplier	Catalog Number
Anti-mouse mouse mast cell protease-6(mMCP6)	R&D Systems®	AF555
Anti- mouse IL-1 alpha	Abcam	ab7632
Anti-human tryptase	R&D Systems®	AF2370

Anti-NF-κB	Abcam	Ab19870
Anti-PAR-2 (SAM11)	Santa Cruz Biotechnology, Inc.	sc-13504
Mouse IgG_{2a}	R&D Systems®	MAB003
Normal goat IgG control	R&D Systems®	AB-108-C

Table 3. List of materials

Material	Supplier	Catalog Number
Biosphere® Filter Tips 0.5-20 µL 2-100 µL 100-1000 µL	Sarstedt	 70.1116.210 70.760.212 70.762.211
Cell strainer, 40 µm	BD Falcon™	352340
Cell strainer ,100 µm	BD Falcon™	352360
Culture flask T 75 T 175	Cellstar®, Greiner-Bio	 658175 660175
Conical tube ,15 mL	BD Falcon™	352096
Conical tube ,50 mL	BD Falcon™	352070
Descosept AF	Dr Schumacher GmbH	sc 311001
LightCycler® Capillaries	Roche	04929292001
Micro tube, 0.5 mL	Sarstedt	72.699
Micro tube, 1.5 mL	Sarstedt	72.690.001
Micro tube, 2 mL	Sarstedt	72.691
Precellys Steel Kit 2.8 mm	Peqlab	91-PCS-MK28
Quality Tips without filter 10 µL 200 µL 1000 µL	Sarstedt	 70.1130 70.760.002 70.762
Serological Pipet 5 mL 10 mL 25 mL	BD Falcon™	 357543 357551 357525
96-well cell culture plate	Cellstar®, Greiner-Bio	655185
Petri dish	Greiner-Bio	632181

Table 4. List of instruments

Instrument	Model	Supplier
Cell counter	CASY® - TTC-2FC-1142	Innovatis AG, Reutlingen
Centrifuge	Megafuge 1.0R	Thermo Scientific, Schwerte
CO_2-Incubater	HERAcell®	Thermo

		Scientific, Schwerte
Electrophoresis System	Sub-Cell® GT	Bio Rad, München
Gel Imager	Gene Genius	Syngene, Cambridge
Inverted Reflected-Light Microscope	Zeiss Axiovert 10	Zeiss, Jena
Light Cycler		Roche, Penzberg
Microplate reader	Dynatech MRX	Dynex Technologies, Chantilly
Multipipette	Multipipette® plus	Eppendorf, Hamburg
Pipette	Eppendorf Reference® / Research®	Eppendorf, Hamburg
Pipettor	Pipetus standard	Hirschmann Laborgeräte, Eberstadt
PCR machine	Px2 Thermal Cycler	Thermo Electron Corporation
Power Supply	POWER PAC 300	BioRad, ,München
Spectrophotometer	Nano Drop 1000	Thermo Scientific, Schwerte
Tabletop centrifuge with refrigeration	Centrifuge 5417C	Eppendorf, Hamburg
Tabletop Centrifuge	Centrifuge 5417R	Eppendorf, Hamburg
Thermomixer	Thermomixer comfort	Eppendorf, Hamburg
Tissue homogenizer	Precellys 24	Bertin Technologies, Montigny-le-Bretonneux
Waterbath	MA6	Lauda, Lauda-Königshofen
Vortexer	REAX 2000	Heidolph, Schwabach

2.2 METHODS

2.2.1 Animal experiments

All experimental procedures were approved by the local State office of Health and Social Affairs and performed in agreement with their protocols. Mice were either purchased from Jackson, Charles River or generated and breed at animal facility of Forschungseinrichtungen für Experimentelle Medizin (FEM).

2.3 MOUSE MODELS

2.3.1 *In vivo* AD model

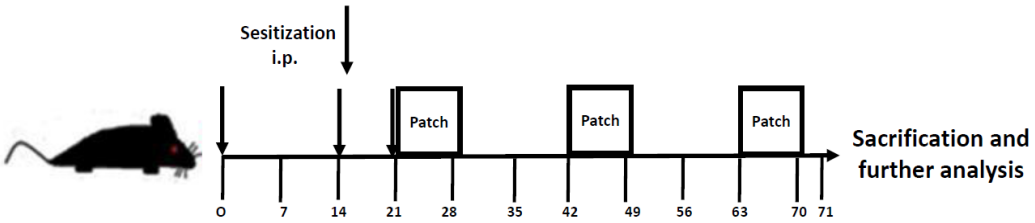


Figure 5. Experimental design for the *in vivo* inducible AD model

AD was induced with an adapted protocol from Dahten *et al.* 2008.[127] Concisely, 10 weels old female C57BL/6 mice with wt, TNF-/-, TSLPR-/- and TNF-/-TSLPR-/- double knockout mice were sensitized using three consequent intraperitoneal injections (i.p) with 100 µl of 10 µg ovalbumin (OVA) adsorbed to 1.5 mg Al(OH)3 (alum) on days 1, 14 and 21 (black arrow on figure 1). The belly of mice was wet shaved on day 21. Followed by tape stripping and epicutaneous application of 100 µg OVA allergen through utilizing patch test method for a period of one week. Three allergen exposures, each of one week duration was given to each mice at the same site on the skin with a two week periods interruption in between without any allergen. On day 71, mice were anesthetized by using isoflurane followed by sacrifcation by cervical dislocation. Blood was collected and 5 mm^2 biopsies were taken for further analysis by ELISA and immunohistochemistry respectively. Photographs of the path area were taken for assessment of severity score. Skin biopsies from the patched skin area were embedded in O.C.T. compound or in formalin for further processing by immunohistochemistry. Rest of the skin was frozen for mRNA isolation and whole skin lysate preparation. All frozen samples were stored at -80 °C unless used for further analysis.

2.3.2 *Ex vivo* skin irritation model

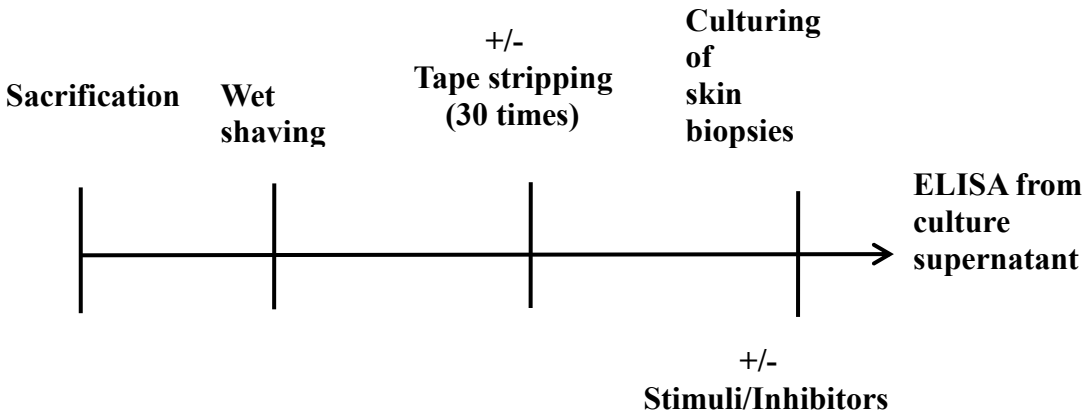


Figure 6. Experimental scheme of skin irritation model with different treatments *ex vivo*.

10 week old female C57BL/6 (wt) mice were anesthetized and sacrified, wet shaved at belly region and exposed 30 times tape stripping using cello tape or left non-irritated. 5 mm^2 punch biopsies were taken. Tape stripped skin biopsies were incubated in hydrocortisone hemisuccinate-free medium with recombinant interleukin-1-receptor antagonist (IL-1Ra) at 200 ng/ml and/or anti-PAR-2 antibody (or mIgG2a control) at 5 µg/ml. Skin explants without tape stripping were exogenously stimulated with recombinant mouse interleukin (IL)-1α (100 ng/ml)and/or PAR-2-Ag (2-Furoyl-LIGRLO-amide, 500 nmol/L). After 12 hrs of incubation, culture supernatant were collected and used for ELISA.

2.3.3 In vivo skin irritation model

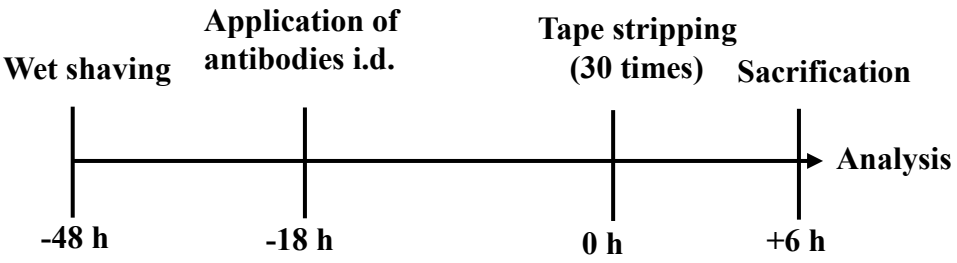


Figure 7. Experimental scheme of skin irritation model with different treatments *in vivo*.

***In vivo* skin irritation**

The experimental procedure is depicted in figure 7. After scarification, mouse skin was chopped and homogenized by pre-chilled precellys homogenization (PEQLAB, Erlangen, Germany) in lysis buffer (2 mmol/L EDTA, 1 mmol/L dithiothreitol, 25 mmol/L Tris [pH 7.8], 1% Triton X-100 and 10% glycerol) with protease inhibitor cocktail (Roche, Basel, Switzerland) and used for ELISA. Protein was quantified by bis-cinchinonic acid (BCA) assay (Pierce Laboratories, Rockford, USA).

2.3.4 Ex vivo mast cell degranulation model

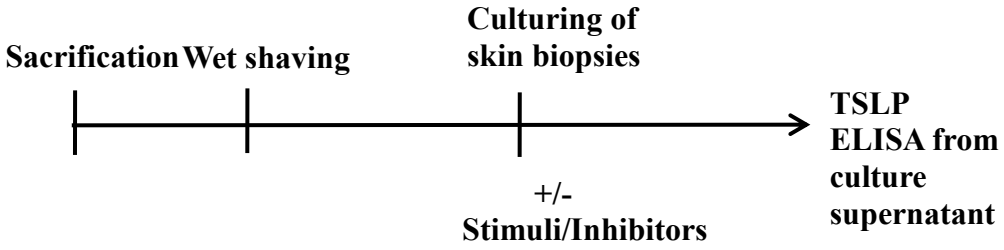


Figure 8. Scheme showing mast cell degranulation model *ex vivo*.
Mice were anesthetized and sacrified and gently wet shaved at belly region. 5 mm^2 punch biopsies were taken and incubated in hydrocortisone hemisuccinate-free medium either with different doses of tryptase or histamine or mast cell supernatant (1%) or mMCP6 (10 ng/ml) or with C48/80 (100µg/ml) for stimulation. For the inhibition skin explants were exposed to mast cell supernatant for 30 minutes prior to treatment with anti-mMCP6 (5 µg/ml) or anti-PAR-2 (5 µg/ml) antibodies. As a control, skin biopsies were either left untreated or treated with respective isotype control antibody (goat-IgG or mouse-IgG). Culture supernatant were collected and TSLP was measured by ELISA after 12 hr of incubation.

2.3.5 In vivo mast cell degranulation model

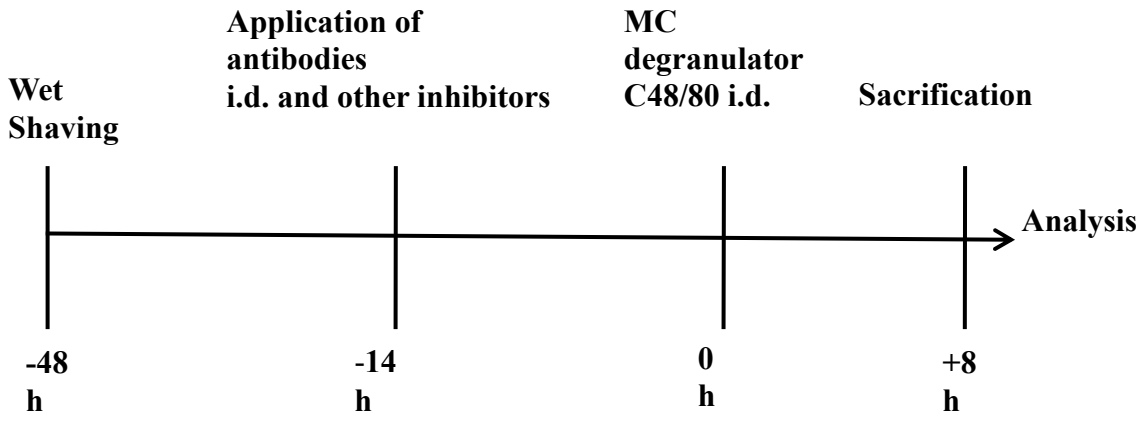


Figure 9. Scheme depicting mast cell degranulation model with different treatments *in vivo*.

The experimental procedure is depicted in figure 9. After scarification, mouse skin was chopped and homogenized by pre-chilled precellys homogenization (PEQLAB, Erlangen, Germany) in lysis buffer (2 mmol/L EDTA, 1 mmol/L dithiothreitol, 25 mmol/L Tris [pH 7.8], 1% Triton X-100 and 10% glycerol) with protease inhibitor cocktail (Roche, Basel, Switzerland) and used for ELISA. Protein was quantified by bis-cinchinonic acid (BCA) assay (Pierce Laboratories, Rockford, USA).

2.4 CELL CULTURE METHODS

2.4.1 Isolation, culturing and treatment of human primary keratinocytes

Human KCs were isolated from foreskin and processed as described previously. The fore skin was obtained after circumcisions, with informed consent of the patients and approval by the university Ethics committee. All the experiments were conducted according to the Declaration of Helsinki Principles. After the 2nd passage, $7.5x10^3$ cells/well were seeded in a 96-well plate in KC medium and grown to 70-80% confluence. For Luciferase assay 40-50% confluent cells were used. After reaching confluence, the medium was changed to hydrocortisone hemisuccinate free KC medium for 24 hr, and cells were stimulated with various concentrations of IL-1(2,5,10,20 ng/ml), nafamostat 5 µg/ml, rhIL-1Ra 200 ng/ml, SLIGRL 100 µM/ml and 0.1% MC lysate 24 hr. Supernatants were collected and measured by a human TSLP ELISA Kit (R&D Systems).

2.4.2 Enzyme linked immunosorbent assay (ELISA)

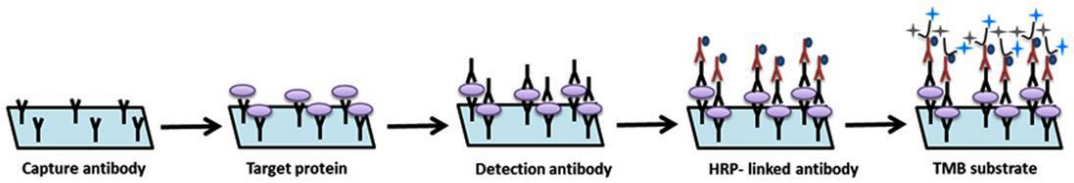


Figure 10. Scheme of sandwich based - enzyme linked immunosorbent assay (ELISA) (adapted from Epitomics - an Abcam Company).

ELISA is an enzyme immunoassay used to measure the unknown level of antigens in serum or supernatant. In this study we have used sandwich based ELISA to quantify the level of protein. Here, first the primary antibodies were coated on the surface of the plate and the

target protein from serum or supernatant were incubated for specific binding. The detection antibodies were incubated over the surface of bound specific antigen. In the next step, the plates were incubated with Horseradish peroxidase (HRP) linked biotinylated antibodies, which can convert a chromogenic substrate. The enzymatic reaction leads to the color change which was measured by spectrophotometer. The concentration of protein in the samples was calculated by the means of standard curve. All the steps were performed at room temperature and in dark from HRP-linked antibody.

2.4.2.1 Mouse and human TSLP ELISA

In vitro, ex vivo, or *in vivo* experiments were performed and cell free supernatant or serum from mice and human epidermal sheet were obtained and measured for mouse and human TSLP levels. Analysis was performed based on TSLP ELISA kit from R&D system (mouse) and ebiosciences (human) according to manufacturer's instructions.

2.4.3 RNA isolation

Frozen skin samples from mice were homogenized by pre-chilled precellys homogenisation (PEQLAB, Germany) beads tube in 500 µl RA1 lysis buffer (NucleoSpin® RNA isolation kit) with 5 µl β-mercaptoethanol (β-Me) at 5500 rpm for 2 times for 30 sec with 5 sec pause. Homogenized samples were transferred to NucleoSpin filter and centrifuged at 11,000 g for 2 min at room temperature. Supernatant was transferred to sterile eppendorf tubes carefully without disturbing the pellet and 500 µl of RNase-free water was added with 10% proteinase K and mixed well for tissue digestion. The lysate was incubated for 15 min at 55 °C. After 15 min, lysate was centrifuged at 10,000 g for 3 min. For RNA isolation from the keratinocytes lysis was performed by using 300 µl RA1 buffer with 3 µl β-mercaptoethanol (β-Me). Further, RNA isolation was performed according to manufacturer's instruction along with DNase digestion step for 15 min at room temperature. RNA was eluted with 60 µl of RNase-free water. Using NanoDrop UV-Vis spectrophotometer, RNA concentration was measured at 260 nm. Later, quality of RNA was checked by 2% agarose gel. The eluted samples were stored at -80 °C for further analysis.

2.4.4 Reverse transcription

Reverse transcription of total RNA into single stranded cDNA was performed with TaqMan® reverse transcription reagent according to manufacturer's instruction. The kit contains a recombinant Moloney Murine Leukemia Virus Reverse Transcriptase, random hexamers and oligo d(T). 1 µg of total RNA was used for reverse transcribtion in to cDNA in thermo cycler with following steps as given in table 5.

Table 5. cDNA synthesis program

Process	Temperature (°C)	Time (min)
Incubation	25	10
Reverse transcription (RT)	48	40
RT inactivation	95	5

All cDNA samples were stored at -20 °C.

2.4.5 Real-time polymerase chain reaction

Fluorescence based real time quantitative polymerase chain reaction (qPCR) was performed on the cDNA for the quantification of gene expression in skin samples or keratinocytes. qPCR was performed with LightCycler® FastStart DNA Master SYBR Green I (Roche) according to the experimental protocol shown in table 6. The cDNA was pre-diluted 1:3 and the primers used were designed by Primer3 software and are given in table 7. The formation of PCR product is measured by increased level of fluorescence caused by specific binding of SYBR green fluorescence dye to double-stranded DNA (SYBR green- Double-Stranded DNA (dsDNA)). To ignore the non-specific binding by SYBR green, PCR buffer also contains a reference dye to normalize the specific binding. The cycle number of crossing point (CP) or the threshold cycle value (CT) is the number of cycle at which significant increase of the normalized florescence is first measured. Depending on CT values of a gene and the efficiency of primers, the relative expression of a gene was calculated. The expression level of target gene was normalized to the expression level of housekeeping gene i.e hypoxanthine-guanine phosphoribosyltransferase (HPRT) using the 2-ΔΔCT method.

Table 6. Real-time polymerase chain reaction protocol

Reagent	Volume/sample (µl)	Final concentration
10XFastStartDNAMasterSYBR Green 1	0.50	1X
25mM MgCl2	0.80	3-5 mM
10µM Forward Primer	0.25	100-500 nM
10µM Forward Primer	0.25	100-500 nM
RNase-free H2O	(makeup the volume up to	

	3µl)	
cDNA	2	

Table 7. Primer sequences used for real time polymerase chain reaction

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	Annealing Temperature
mTSLP	AGAGAAGCCCTCAA TGACCA	GGACTTCTTGTG CCATTTCC	61 ºC
mHPRT	CGTCGTGATTAGCG ATGATG	AATCCAGCAGGT CAGCAAAG	60 ºC
hShort TSLP	CCTGAGTAGCATTTA TCTGAG	CCGCCTATGAGC AGCCAC	62 ºC
hLong TSLP	TAGCCTGGGCACCA GATAGC	CACCGTCTCTTG TAGCAATCG	60 ºC
hHPRT	CGTCGTGATTAGCG ATGATG	AATCCAGCAGGT CAGCAAAG	60 ºC

2.4.6 Immunoprecipitation assay (IP):

For immunoprecipitation of tryptase, the cells were lysed in PBS containing protease inhibitors using freez-thaw cycles. The lysates were diluted twenty-fold with PBS and incubated with protein G beads (Santa Cruz Biotechnology) and human anti-tryptase polyclonal antibody (R&D systems) or goat IgG isotype control. Immunoprecipitated mast cells (MCs) lysate was collected, re-suspended in sample buffer and analyzed by immunoblotting.

2.4.7 Immunoblotting:

The MC lysates, immunoprecipitated with human anti-tryptase antibody or goat IgG isotype control, were suspended in sample buffer, loaded onto 10% polyacrylamide gels, and transferred to polyvinylidene difluoride membranes. The membranes were probed with anti-tryptase antibody, detected using appropriate secondary antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology), and enhanced with a chemiluminescent kit (Pierce).

2.4.8 Chromatin Immunoprecipitation (ChIP) assay:

Keratinocytes (1.2 x10^5 cells/condition) were stimulated for 30 minutes using IL-1 and/or PAR-2 agonist. Chromatin was prepared using Chromatrap Enzymatic Shearing Kit (Chromatrap) as per manufacturer instructions and the chromatin resolved on a 1% agarose gel for size confirmation and shearing quality. The chromatin was further processed using Abcam high sensitivity ChIP kit (Abcam). Immunoprecipitation was carried out using anti-NF-κB antibody, and rabbit IgG as control. After column purification DNA was analysed using SYBR Green PCR kit and the rotor gene thermocycler (both from Qiagen). ChIP DNA was normalised to that of input DNA. For the human IL-8 promoter region EpiTect ChIP qPCR primers were purchased from Qiagen. The sequences of primers and conditions are as specified in table 8.

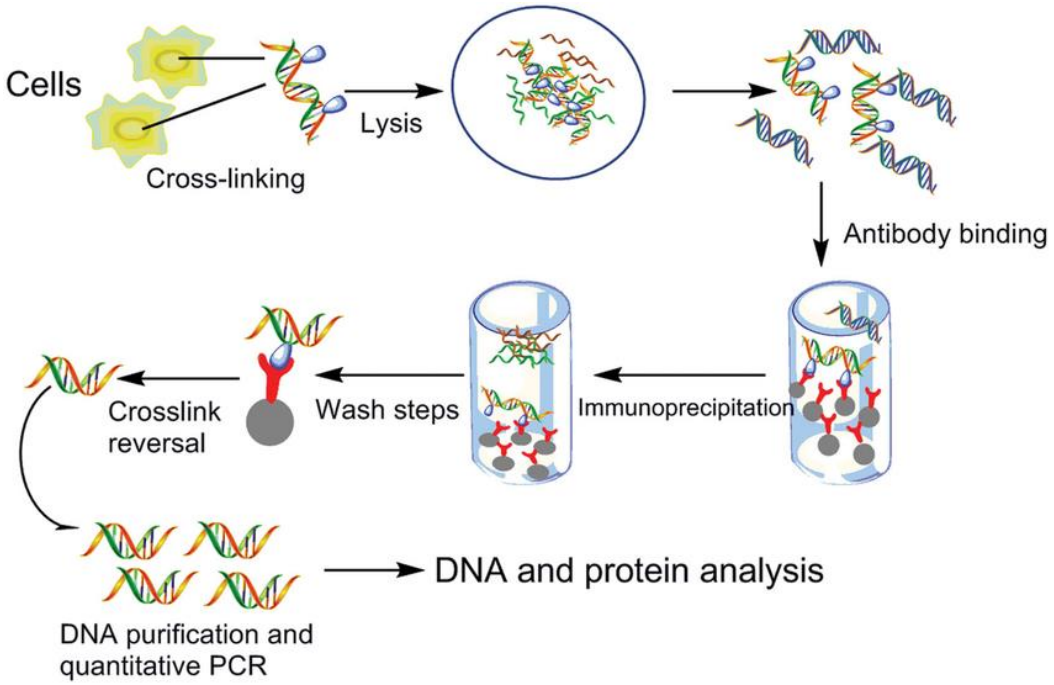


Figure 11. Graphical depiction of ChIP assay. Protein and associated chromatin in living cells are crosslinked using formaldehyde. The crosslinked DNA–protein complexes (chromatin-protein) are then sheared into ~500 bp DNA fragments using either enzymatic digestion or physical shearing by sonication. The DNA-protein complexes are then immunoprecipitated by an appropriate protein-specific antibody. After the cross-links are reversed, the associated DNA fragments are eluted, which is followed by

immunoprecipitation of the crosslinked complexes and analysis of the resultant DNA by qPCR. **(adapted from Sigma-Aldrich)**

Table 8. Sequences of primers used for chromatin immunoprecipitation

Region	**Forward Primer (5′-3′)**	**Reverse Primer (5′-3′)**	**Annealing Temperature**
NF-κB BS1 hTSLP promoter	GGGCAAAGCAA AAAGGAGGAA	TAAACGCCTA CGGGCTCTTTT	60 ºC
NF-κB BS2 hTSLP promoter	CATTTGCTGGGA CTCACACAC	CTGCATCGCTC TGGTCCTT	55 ºC
NF-κB BS3 hTSLP promoter	ATTTTCTGGTCC TTCACATGGGT	TGTCCAAATG CTGAAGGAGT G	60 ºC
NF-κB BS4 hTSLP promoter	GGAGGGTCCAG AGCAATACAC	GGGGGAGATC TGTCTCTTTGG	60 ºC
GAPDH promoter	TAGGGCCCGGC TACTAGCGGTTT TA	AGCCAGTCCC AGCCCAAGGT CTTGA	62 ºC
Beta-Actin promoter	AAATGCTGCACT GTGCGGCGAAG	CGAGCCATAA AAGGCAACTT TCGGA	63 ºC

2.4.9 Plasmid Construction, transfection and luciferase assay:

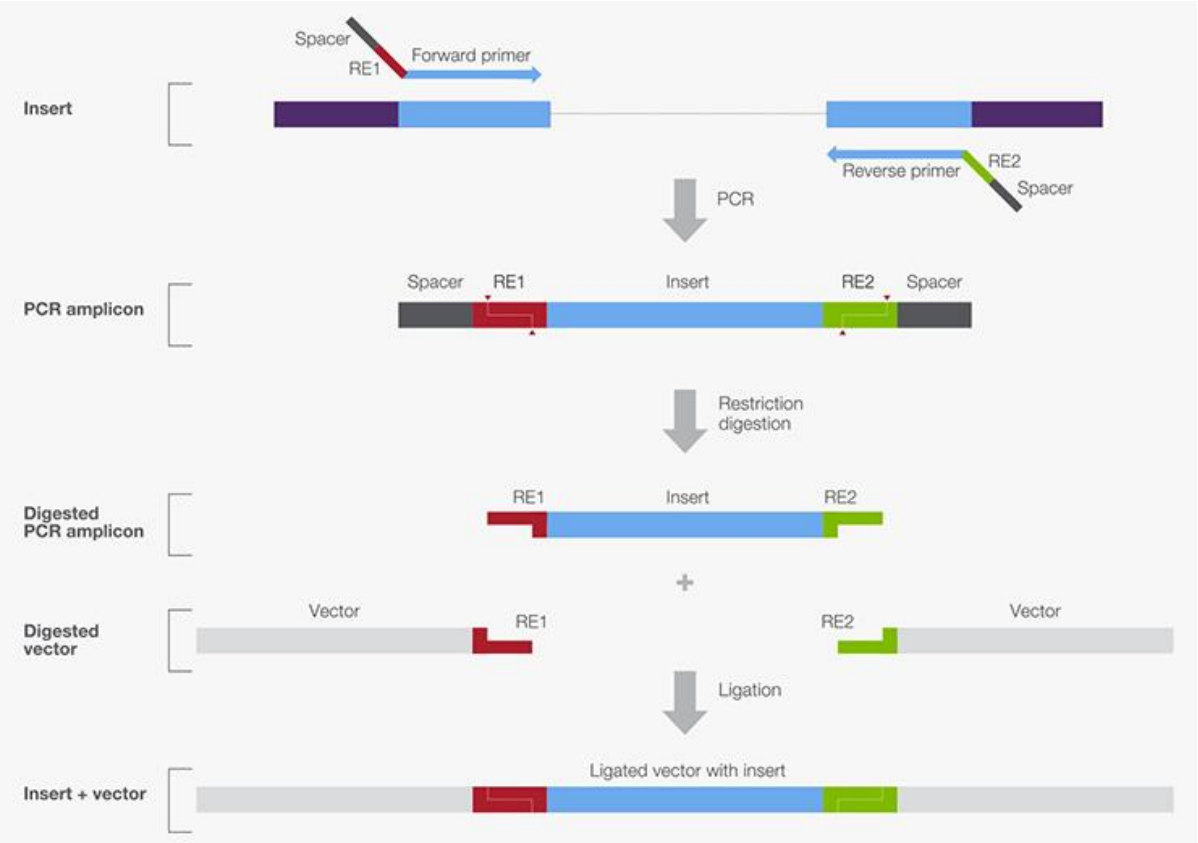


Figure 12. Graphical summary of cloning by restriction enzymes. (reprinted from ThermoFischer Scientific)

Human TSLP promoter fragment was amplified by PCR from genomic DNA isolated from human primary KCs, using Q5 high-fidelity DNA polymerase (New England Biolabs). Kpn1-ended primers, and NheI-ended antisense primers were used to generate the 4.2kb human TSLP fragment. The fragment was cloned into the pGL4.15 basic vector (Promega) using restriction enzyme cloning and its sequence was verified by sequencing.

Keratinocytes (1.2 x10^5 cells/condition) were transfected with 1.6µg of TSLP promoter luciferase reporter plasmid and 0.4µg of the Renilla luciferase reporter vector pRL-TK (Promega) using FuGENE 6 transfection reagent (Promega) according to the manufacturer's protocol. After transfection, cells were cultured for 24 h, then stimulated for 24 h with interleukin (IL)-1α and/or PAR-2-Ag (SLIGRL). Cells were harvested and lysed in 100 µl of lysis buffer (Promega). The luciferase activity was measured by the dual-luciferase assay system kit (Promega) using a VictorTMX3 luminometer (PerkinElmer). All values were normalized to Renilla luciferase activity.

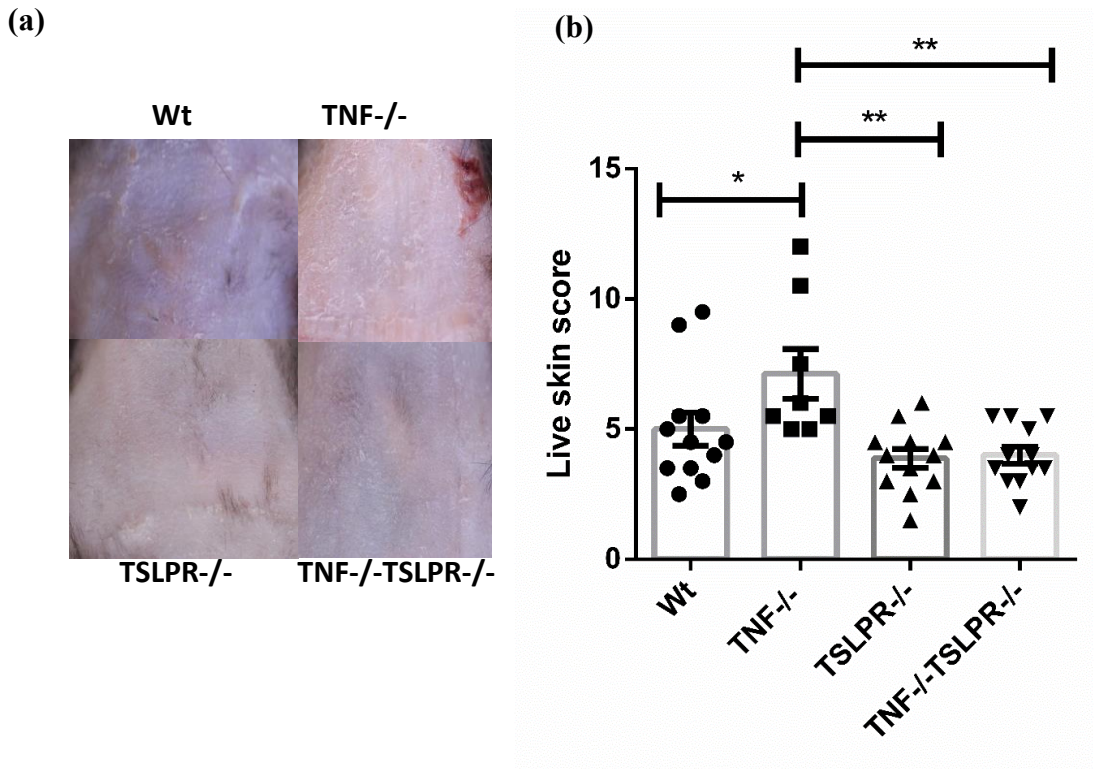
2.5 STATISTICAL ANALYSIS

Normally distributed data are depicted as mean ± SEM and non-normally distributed data are shown as median ± range. Experiments with only two groups were analyzed using *t*-test (paired or unpaired) or Wilcoxon matched paired test, when groups were not normally distributed; for more than 2 groups, depending on the data distribution, 1-way analysis of variance (ANOVA) was used, followed by Bonferroni multiple comparisons test or Kruskal-Wallis test and Holm-Sidak multiples comparison test. Statistical analyses were performed with GraphPad Prism version 6 (GraphPad Software, USA). *P* value less than 0.05 was considered as statistically significant.

3 RESULTS

3.1 TSLPR-/- MICE ARE PROTECTED FROM ENDOGENOUS TNF-DEFICIENCY- MEDIATED AD DEVELOPMENT

Numerous studies have established a role for TSLP as a Th2-promoting cytokine and master switch of atopic dermatitis (AD).[41,128-130] As TNF-α is well established elicitor of TSLP[131] the interaction between TNF and TSLP in the development of AD in mice was investigated. As suspected TNF-/- mice developed profound AD symptoms compared to Wt mice but interestingly TSLPR-/- mice as well as TNF-/-TSLPR-/- were protected from AD development. The three genotypes (Wt, TSLPR-/- and TNF-/-TSLPR-/-) showed significantly



less symptoms of AD compared to TNF-/- mice (Fig. 13 a, b).

Figure 13. **Protection of AD in TSLPR-/- mice in the absence of TNF.** (a) AD development is suppressed in TNF-/-TSLPR-/- double knockout mice (b) symptom score which represents a characteristic of dermatitis. Data was analyzed using one way ANOVA with Holm-Sidak multiple comparison test. Mean ± SEM of n = 8-12 mice/group. (*$p<0.05$, **$p<0.01$).

These results show that TNF deficiency predisposes to AD in a TSLP dependent manner. In contrast, at the baseline the skin of TNF-/- mice was normal and healthy as Wt, TSLPR-/- and TNF-/-TSLPR-/- genotypes with comparable MC number, T cells, epidermal and dermal thickness (data not shown), indicating that the skin structure does not require TNF-α and/or TSLPR for development and maintenance.

3.2 SKIN IRRITATION INDUCES TSLP PRODUCTION IN MURINE SKIN *EX VIVO*

3.2.1 Skin irritation induces TSLP production in murine skin by IL-1 and PAR-2 dependent pathways *ex vivo*

Various studies have shown that skin barrier disruption either by genetic manipulations or physical/chemical irritation can induce robust TSLP expression[20,36,122,123]. Tape stripping, as a method for inducing physical irritation mediated barrier disruption and is also part of the AD model established in our group. To address the mechanisms behind physical irritation induced TSLP expression, we subjected the murine skin to tape stripping and studied the skin explant *ex vivo* (Fig. 6). As expected, tape stripping mediated barrier disruption induced TSLP production in a time dependent manner (Fig. 14a), consistently with the previous report[20], but now providing kinetic resolution.

As keratinocytes (KCs) express interleukin (IL)-1 and protease activated receptor (PAR)-2[91,132,133] it was hypothesized that disruption of skin barrier triggers activation of IL-1 and PAR-2 pathways concurrently to trigger signaling pathways leading to TSLP induction. To test this, we neutralized the pathways individually as well as jointly by means of interleukin-1 receptor antagonist (IL-1Ra) and a function-blocking anti-PAR-2 antibody (Fig. 14b). Neutralization of PAR-2 or IL-1 pathways alone blunted the TSLP production but blocking of both pathways concurrently resulted in amplified suppression compared to inhibition of either pathway individually (Fig. 14b).

To verify the interaction between PAR-2 and IL-1 in the induction of TSLP, the next reciprocal approach was to activate the two pathways exogenously by providing respective stimuli. It was found, that TSLP production was upregulated upon stimulation with protease-activated-receptor-2-agonist (PAR-2-Ag) as well as recombinant IL-1 (Fig. 14c). Yet, simultaneous activation of both pathways resulted in enhanced TSLP production compared to the response attained with either stimuli individually (Fig. 14c).

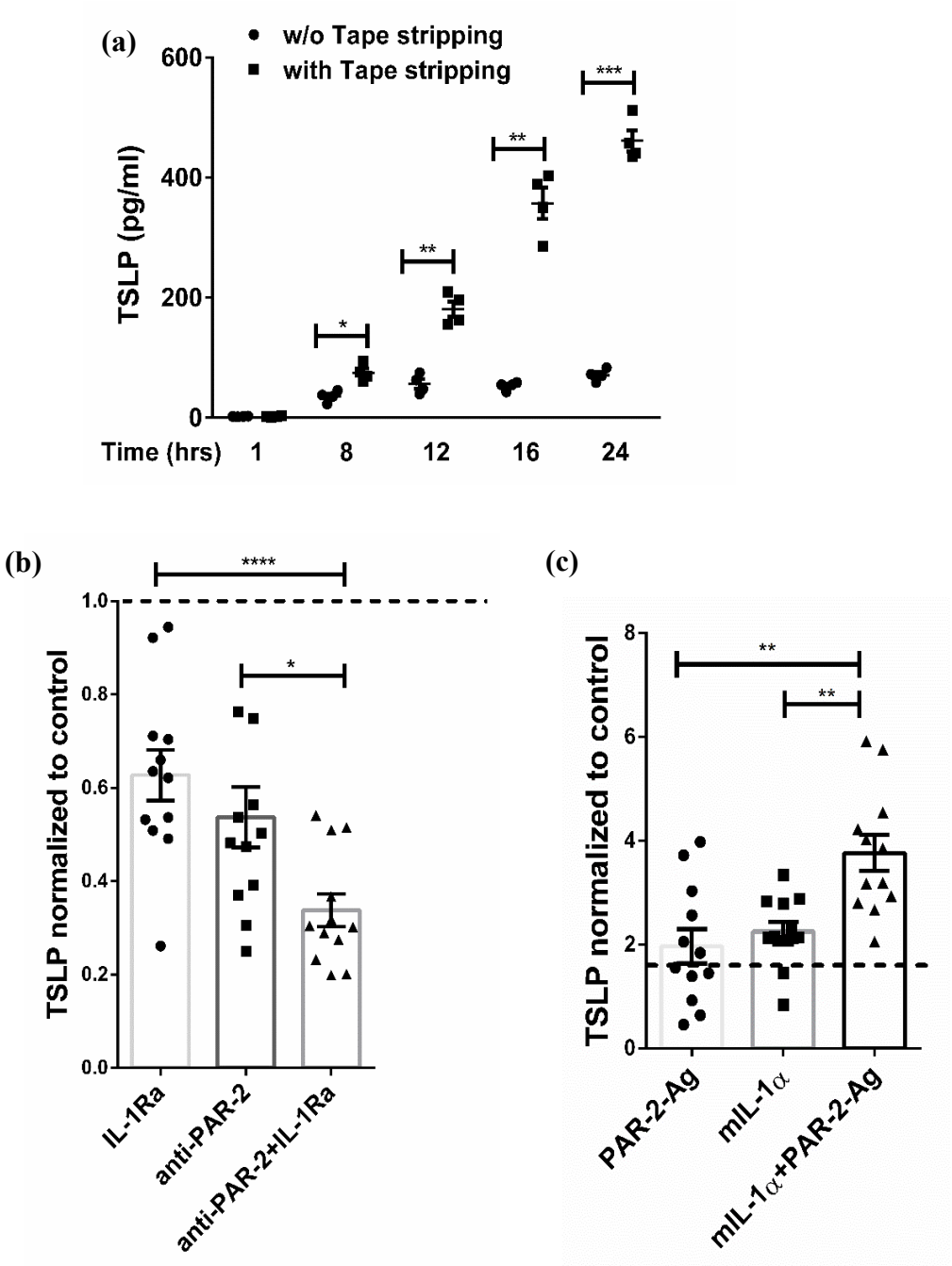


Figure 14. **Skin irritation induces thymic stromal lymphopoietin (TSLP) expression via interleukin (IL-1) and protease-activated receptor (PAR)-2 *ex vivo*.** (a) Kinetics of tape stripping induced TSLP production. TSLP was measured in culture supernatant by using enzyme-linked immunosorbent assay (ELISA) after different incubation times. (b) Murine skin explants after tape stripping, were incubated with anti-PAR-2 antibody (mIgG2a served as the isotype control) or interleukin-1 receptor antagonist (IL-1Ra) either alone or in combination. (c) Naïve murine skin biopsies (i.e. without tape stripping) were incubated with mIL-1α alone or protease-activated receptor-2-agonist (PAR-2-Ag) or in combination. (b) and (c) TLSP was measured by ELISA after 12 h. The data, given as mean ± SEM from 12 independent experiments, was analyzed using (a) paired t-test (n = 4) or (b, c) Kruskall-Wallis

with Dunn's multiple comparison test on the normalized data (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

3.2.2 IL-1 and PAR-2 pathways collaborate in physical irritation mediated TSLP production *in vivo*

As a next step it was investigated whether the *ex vivo* findings are interchangeable to the intact skin *in vivo.* A tape stripping model was performed after the neutralization of IL-1, PAR-2 or both by using function-blocking antibodies. The experimental scheme is shown in figure 7. TSLP abundance in the skin was inhibited by intervention with PAR-2 or IL-1 pathways, while simultaneous inhibition of both pathways resulted in a profound decrease of

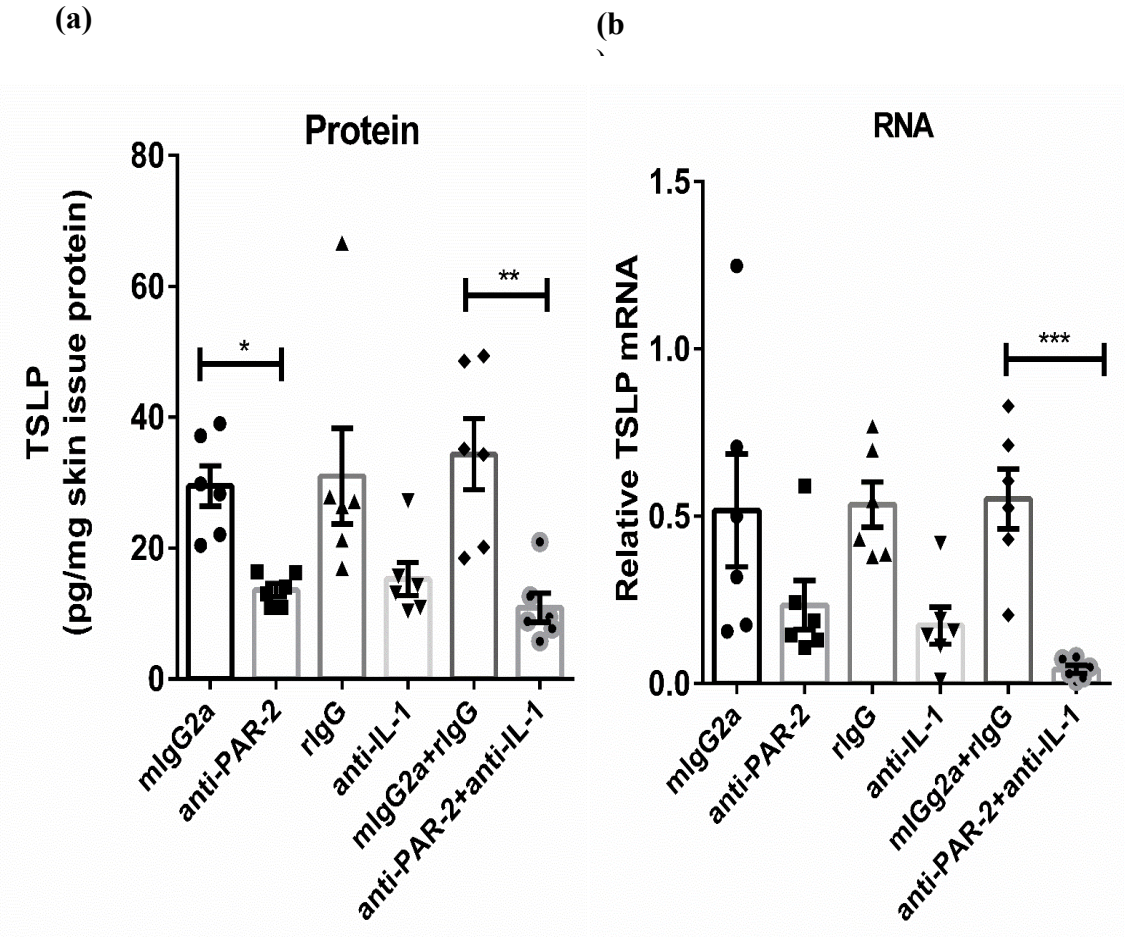


Figure 15. Skin irritation mediated TSLP production is driven by IL-1 and PAR-2 *in vivo*. 20 µg of anti-PAR-2 and/or anti-IL-1 (mIg2a and rIgG served as control) was intradermally injected in each mouse. (a) TSLP was measured in skin lysate using ELISA. (b) mRNA expression of TSLP in murine skin. Data are shown as mean ± SEM of 6 independent experiments and was analysed by Kruskal-Wallis test combined with Dunn's multiple comparisons test (*p<0.05, **p<0.01, ***p<0.001).

TSLP (Fig. 15a). The inhibition at mRNA expression level was similar and TSLP transcript levels dropped to more than 40% of control when IL-1 and PAR-2 pathways were concomitantly blocked (Fig. 15b). These results indicate that irritation mediated TSLP response results from concurrent activation of IL-1 and PAR-2 pathways.

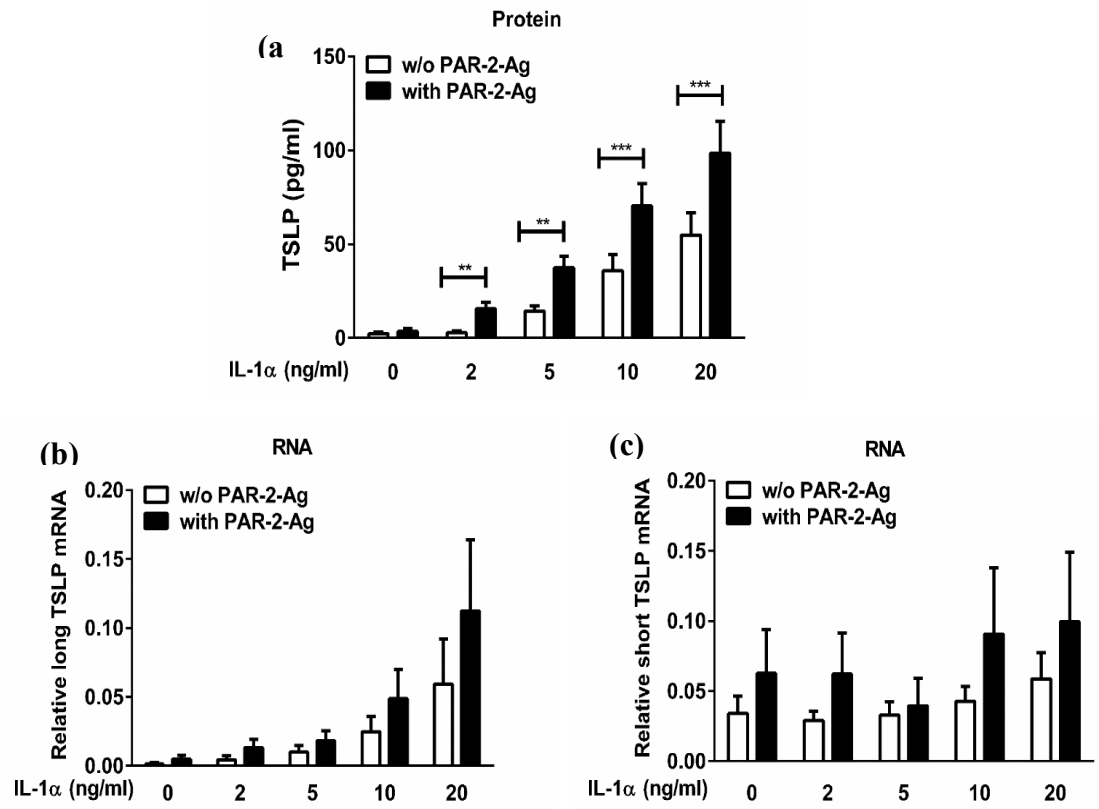
3.2.3 PAR-2 agonist and IL-1 collectively induce TSLP in primary human keratinocytes

To delineate the findings in murine system are intimated in humans, primary human KCs were stimulated with IL-1α (at different concentrations) and PAR-2 agonist alone as well in combinations, and TSLP levels were quantified. In this experimental setting, IL-1α induced TSLP in a dose-dependent manner while stimulation with PAR-2-Ag alone was without effect (Fig. 16a). Furthermore, TSLP induction was more prominent under co-stimulation with PAR-2-Ag and IL-1α and this was seen at all the tested concentrations of IL-1α. An actual synergistic effect was apparent at lower IL-1α concentrations (2 ng/ml), which by its own were unable to induce detectable TSLP response (Fig. 16a).

Two TSLP mRNA isoforms have been reported in humans. The short isoform is constitutively expressed and shown to exert ant-microbial properties whereas long isoform is conjugated to inflammation.[134,135] To examine the influence of IL-1 and PAR-2 pathways activation on the TSLP isoforms, human KCs were stimulated as described above and harvested for RNA extraction after 2.5 h. Actually the long isoform was barely expressed before the stimulation, but was dose-dependently induced by IL-1α (Fig. 16b). The PAR-2 agonist evidently collaborated with IL-1 in the upregulation of the long isoform (Fig. 16b). Contrary to the expression of long isoform, the short isoform was clearly detectable and not significantly modulated by IL-1, PAR-2 or the two jointly although there was a tendency of PAR-2-Ag to augment the transcript (Fig. 16c). Collectively, IL-1 and PAR-2 pathways concertedly trigger TSLP production also in human KCs by facilitating the expression of its long isoform.

3.2.4 PAR-2 and IL-1 pathways converge on the TSLP promoter by concerted recruitment of NF-κB

The above findings indicated that the PAR-2 and IL-1 triggered signaling cascades may merge on the TSLP promoter. NF-κB is the major transcription factor in the transcriptional



activation of TSLP gene.[135] Four consensus binding sites (BS)s have been identified in TSLP promoter (designated BS1-BS4, see Fig. 17a), two of these BSs have been involved in TSLP induction (BS1 and BS4).[131,136,137]

Figure 16. PAR-2 and IL-1 by working together preferentially induces the long TSLP isoform in human keratinocytes. (a) Human primary keratinocytes were stimulated with PAR-2-Ag and/or IL-1α for 24 h. TSLP protein levels were measured by ELISA in culture supernatants after stimulation. (b) Gene expression of the long and (c) short TSLP isoforms after incubation with PAR-2-Ag and/or IL-1α for 2.5 h was quantified by real time quantitative polymerase chain reaction (RT-qPCR). Results are expressed as mean ± SEM from 9 independent experiments and analysed by paired t-test (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

To study NF-κB binding to these BSs under given experimental settings, nuclear extracts were prepared from IL-1α and/or PAR-2-Ag treated KCs and ChIP assays were performed. Recruitment of NF-κB was augmented by KC stimulation at BS1 and more potently at BS4

(Fig. 17b,c), but not at BS2 or BS3 (Fig. 18). Combined stimulation with IL-1α and PAR-2-Ag resulted in concerted recruitment. Specially, NF-κB binding to BS4 was barely detectable upon IL-1 or PAR-2 activation alone yet became profound when the signaling cascades were simultaneously triggered (Fig. 17c).

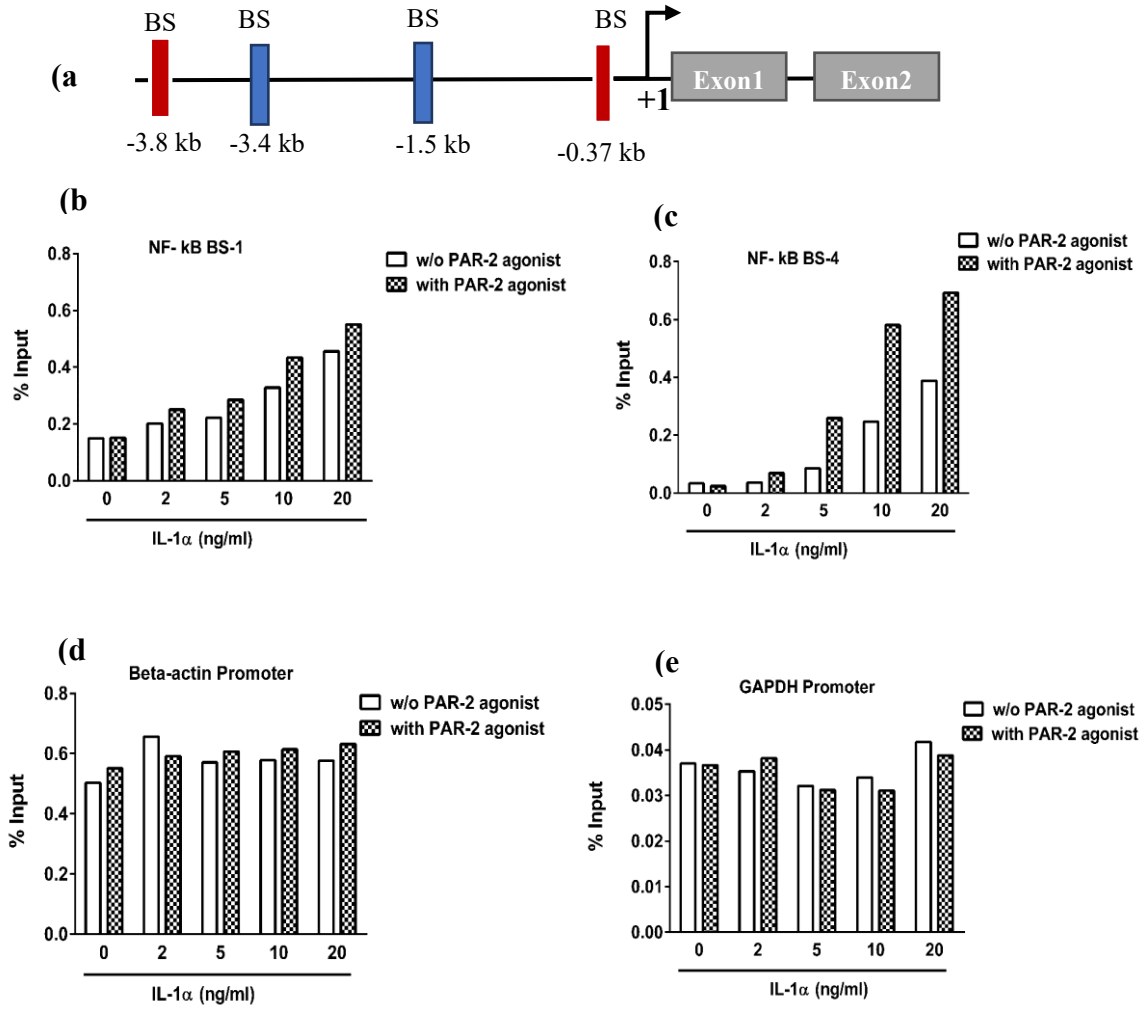


Figure 17. **NF-κB binding to the TSLP promoter is promoted by IL-1 and PAR-2.** (a) Diagrammatic representation of the consensus NF-κB binding sites in -3.8kb region of the human TSLP promoter. (b) Recruitment of NF-κB to BS1 and (c) Recruitment of NF-κB to BS4 of the human TSLP promoter upon stimulation with PAR-2-Agonist (PAR-2-Ag) and/or IL-1α. (d, e) Beta-actin and GAPDH promoter regions were used to verify that PAR-2-Agonist (PAR-2-Ag) and/or IL-1α stimulations do not alter binding affinity of RNA polymerase II to meaningful regions of housekeeping genes. Results from one representative experiment of 3 independent experiments are shown.

To rule out non-specific effects of the according treatments, the 5'-upstream regions of beta-actin and GAPDH were used as controls. As expected, RNA polymerase II binding to upstream regions of these genes was comparable across conditions in accordance with housekeeping-like character of the genes. In addition, the validity of the ChIP approach was confirmed by using an isotype control antibody comparing it directly with the specific antibody in different conditions (Fig. 18). To further validate the specificity of the ChIP antibody, the well-documented NF-κB-BS from the IL-8 promoter[138] was used as a positive control for activation-dependent NF-κB binding (Appendix Fig. 29).

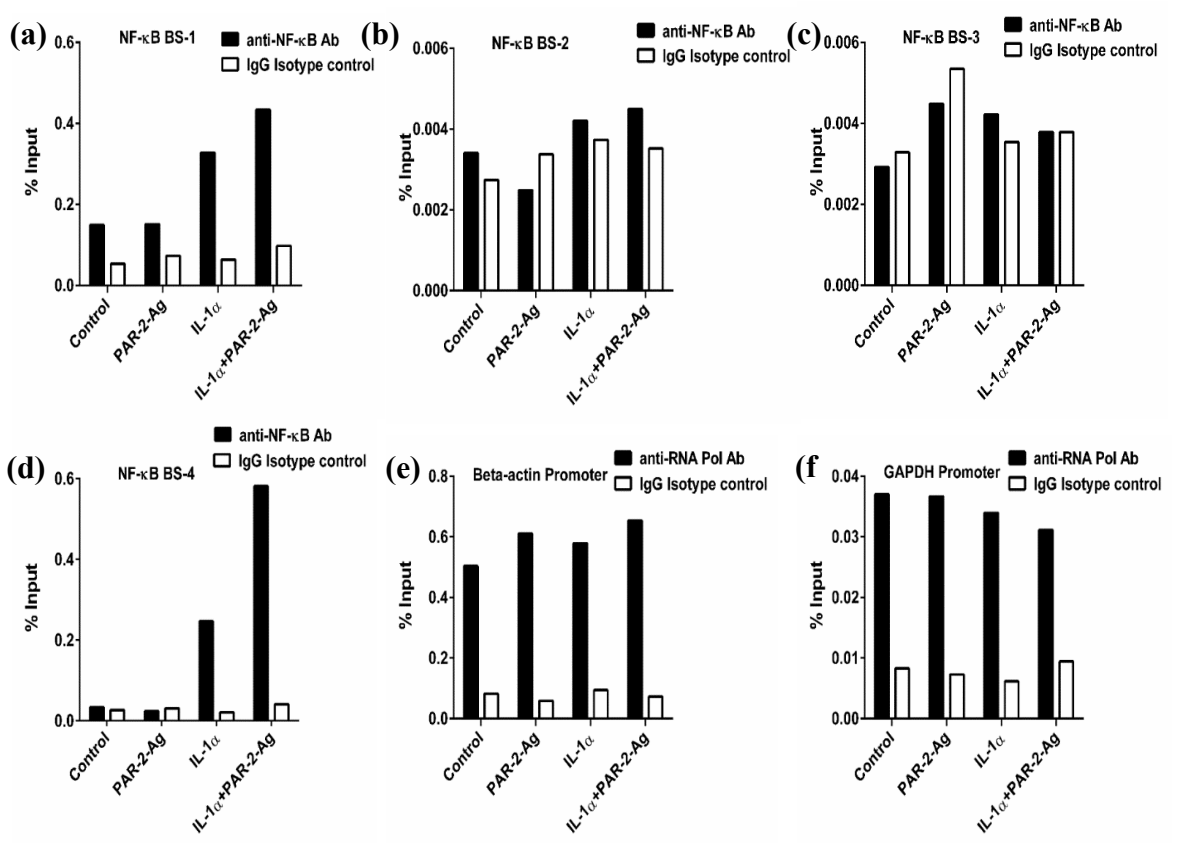
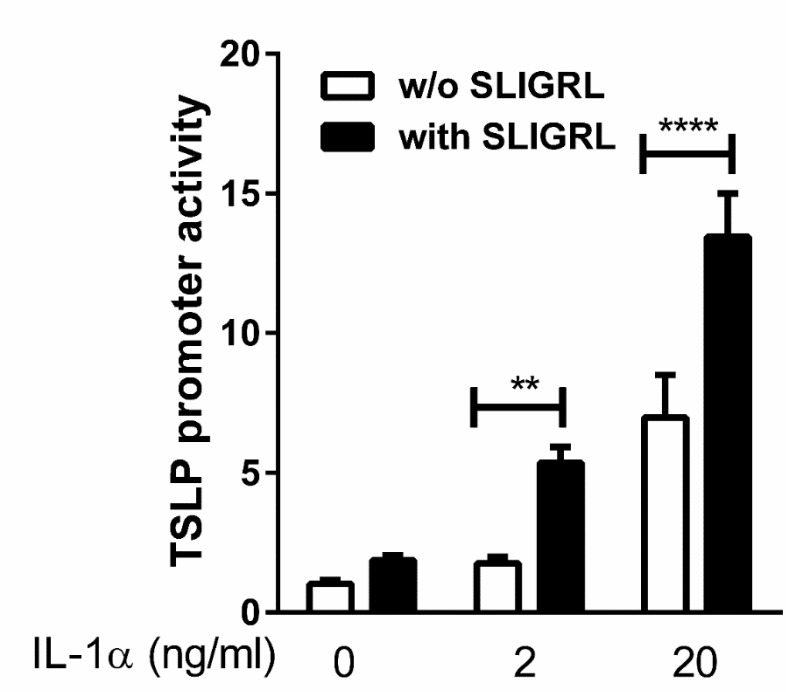


Figure 18. IL-1 and protease activated receptor (PAR-2) activation leads to recruitment of NF-κB to binding sites (BS)s 1 and 4, but not BS2 and BS3 of the human TSLP promoter. Recruitment of NF-κB at BS1 (a), BS2 (b), BS3 (c), and BS4 (d) of the human TSLP promoter region, in comparison to IgG isotype control antibody. Promoter regions of (e) Beta-actin and (f) GAPDH upon stimulation with PAR-2-Ag and IL-1α (10 ng/ml) in comparison to IgG isotype control antibody. Results from one representative experiment of 3 independent experiments are shown.

Taken together, the presented results suggest that PAR-2-and IL-1-mediated TSLP induction is triggered by their joint activity at the TSLP promoter through collaborated recruitment of NF-κB to its most biologically meaningful binding site.

3.2.5 PAR-2 and IL-1 induce transcriptional activation of the TSLP promoter in human keratinocytes

To investigate the role of IL-1 and PAR-2 in the activation of the TSLP promoter, was employed transient transfection of sub-confluent human primary KCs with a proximal 4.2 kb construct containing the transcriptional start of human TSLP promoter fused to a firefly luciferase gene. The response to IL-1 and/or PAR-2Ag was quantified by measuring luciferase activity. At baseline, little promoter activation was observed. PAR-2Ag or low concentration of IL-1 (2 ng/ml) alone showed no induction of TSLP promoter activity against control but the combination of both stimuli resulted in profound activation (Fig. 19), emphasizing the hypothesis that both pathways merge at the TSLP promoter. At a higher concentration IL-1 (20 ng/ml) was able to increase the TSLP promoter activity on its own, but the combined stimulation with PAR-2-Ag further enhanced the activity. Collectively, these results further support an important role of the PAR-2 signaling cascade in the co-activation



of the TSLP promoter, particularly in the presence of low levels of IL-1.

Figure 19. IL-1 and PAR-2 mediated transcriptional activation of the human TSLP promoter. Primary human KCs were transfected with 1.6 μg of luciferase (Firefly) reporter

plasmid containing the human TSLP promoter and 0.4 µg of the Renilla luciferase reporter vector pRL-TK. After 24 h of transfection, KCs were stimulated with IL-1 and/or PAR-2-Ag for 24 h. Whole-cell lysate was prepared and luciferase activity was measured. Renilla luciferase activity was used for normalization. Data are given as mean ± SEM from 9 independent experiments and was analyzed by Kruskal-Wallis test combined with Dunn's multiple comparisons test (**p<0.01, ****p<0.0001).

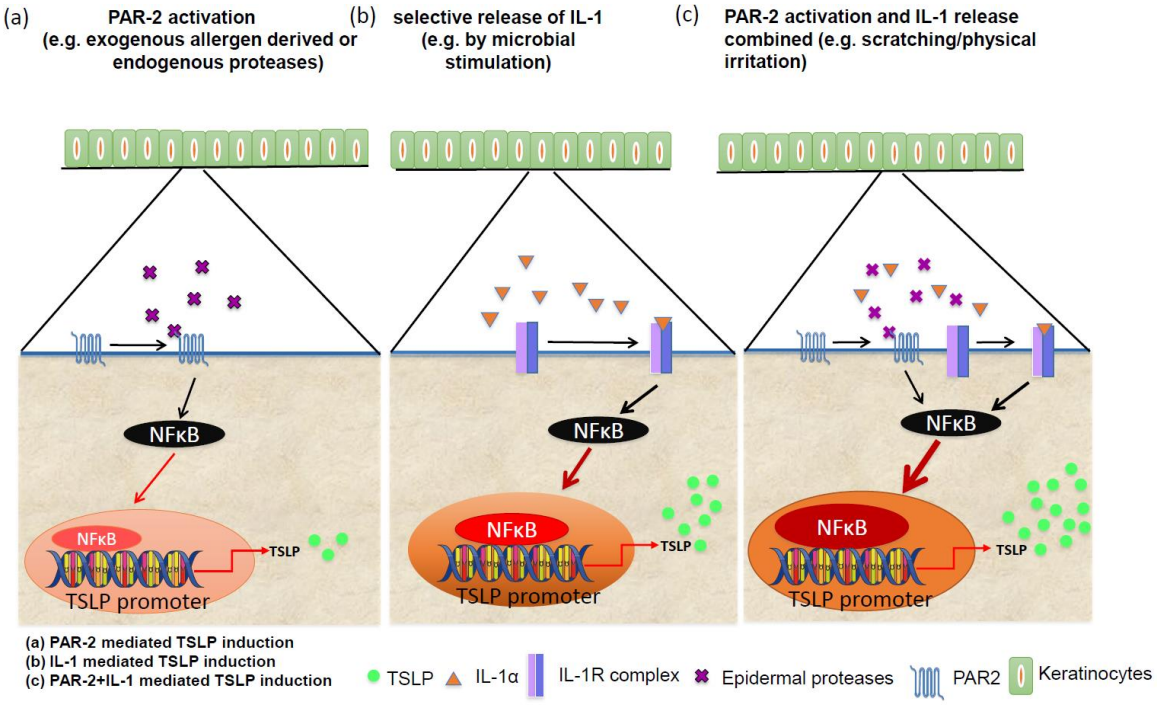


Figure 20. Graphical depiction of the mechanism of skin irritation mediated TSLP induction. (a) PAR-2 pathway activation leads to a moderate enhancement in NF-κB activity, (b) while IL-1 pathway activation alone has a more intense effect on this signaling route in comparison to PAR-2. (c) Yet, upon combined activation of the IL-1 and PAR-2 pathways, by physical skin irritation, leads to an amplified signal and promoted promoter activity of NF-κB; this results in profound TSLP production and release after transcription in comparison to each stimuli alone.

3.3 MAST CELLS CONTRIBUTE TO TSLP PRODUCTION

3.3.1 Murine skin produces TSLP in a mast cell tryptase and PAR-2-dependent mechanism *ex vivo*

Since AD is mainly driven by TSLP overproduction and mast cells (MCs) have been associated with TSLP in various contexts[121] such as MCs express highest levels of TSLPR as revealed by body-wide expression atlas for humans (FANTOM5) and also responsive to

TSLP. Furthermore, MCs also enhance TSLP production by epithelial cells[121,139]. Based on literature and data produced in our group it was hypothesized that MCs act as intermediaries in the biological cascade promoting TSLP production, thereby boosting AD. To address the mechanisms behind MCs induced TSLP expression, murine skin was treated with purified MC mediators as well as MCs supernatant and the skin explant was studied *ex vivo* (Fig. 21). The results give strong evidence that MCs products specifically tryptase is crucial for the enhanced TSLP production (Fig. 21a) by murine skin explants, while histamine showed no impact on TSLP production (Fig. 21b). Murine skin explant produced excessive TSLP in response to tryptase in a dose dependent manner (Fig. 21a), while being neutral to histamine dose response (Fig. 21b). To study the mechanism behind the tryptase mediated exaggerated TSLP production by murine skin, it was investigated whether the murine skin was prone to enhanced TSLP production also upon exposure to MC supernatant, or whether the boost in TSLP production requires purified tryptase. As expected, the presence of MC supernatant induced robust TSLP response in murine skin *ex vivo*. MC supernatant mediated enhanced TSLP induction was significantly inhibited by anti-mMCP6 antibody (Fig. 21c).

As shown earlier, PAR-2 was involved in skin irritation-mediated TSLP production, therefore it was asked whether the mediators in the MC supernatant act via PAR-2. To investigate this, murine skin was exposed to the MC supernatants followed by anti-PAR-2 antibody. The results demonstrated that endogenous PAR-2 is essential for the MC supernatant mediated TSLP *ex vivo*, as anti-PAR-2 antibody drastically inhibited the TSLP production (Fig.21d). To validate the interplay between tryptase and PAR-2 during the induction of TSLP, the next complementary approach was to stimulate Wt and PAR-2-/- murine skin explants exogenously by providing mMCP6 (murine equivalent of human tryptase).

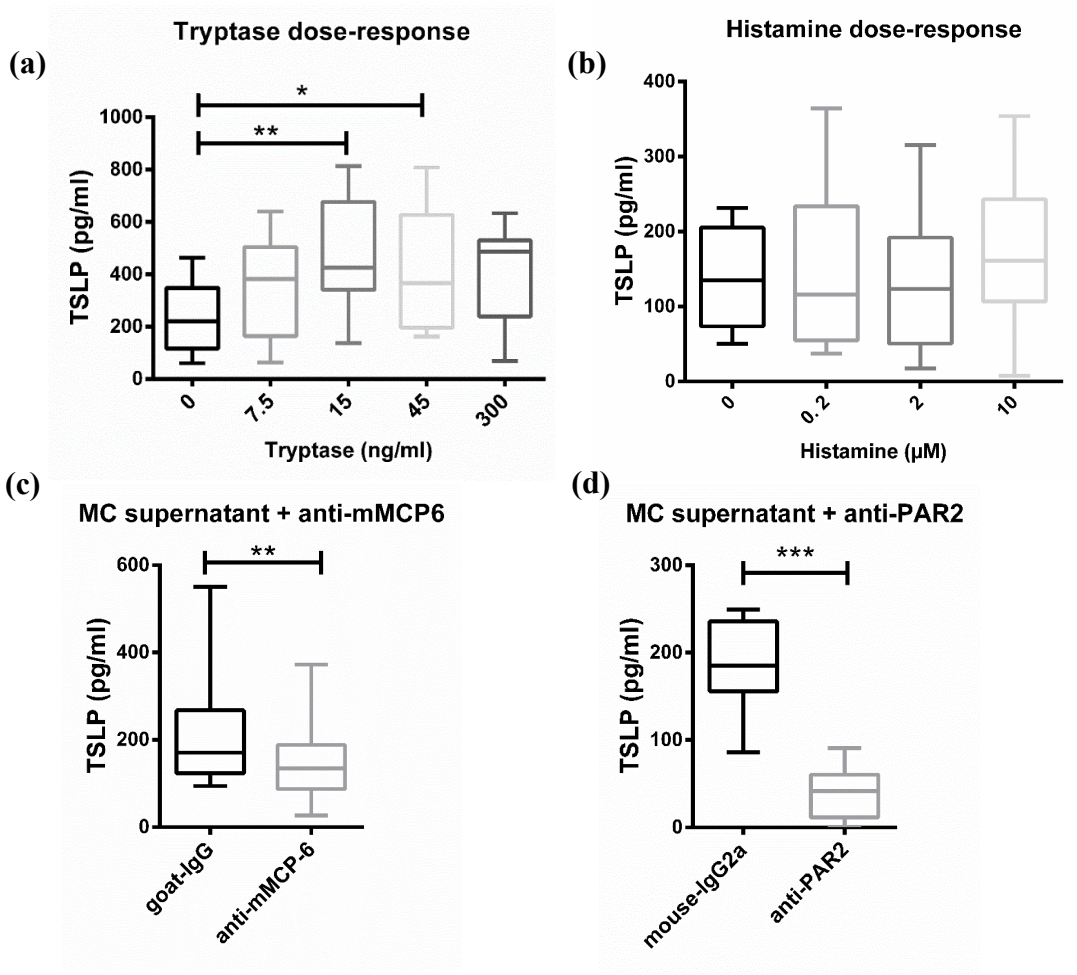


Figure 21. Mast cells instruct murine skin to produce TSLP by a tryptase and PAR-2 dependent mechanism. (a) Dose response of tryptase. (b) Histamine dose response. (c) After stimulation with mast cell supernatant, murine skin explants were incubated with anti-mMCP6 antibody. gIgG served as isotype control. (d) Biopsies of murine skin after stimulation with mast cell supernatant were incubated with anti-PAR-2 antibody (mIgG2a served as isotype control). (a-d) Levels of TLSP were measured by ELISA after 12 h.

The data, given as mean ± SEM, was analyzed using (a, b) Kruskall-Wallis with Dunn's multiple comparison test or (c, d) paired t-test. mean ± SEM from 6-12 independent experiments (*p<0.05, **p<0.01, ***p<0.001).

In line with the previous results, Wt murine skin explants resulted in robust TSLP production upon treatment with mMCP6, while PAR-2-/- skin explants were non-responsive to mMCP6 stimulation (Fig. 22). Upon treatment with mMCP6 Wt skin explants produced significantly higher amount of TSLP compared to untreated Wt skin explants and to their counterpart PAR-2-/- skin explants, while at baseline (unstimulated skin explants) there were no differences in TSLP production across the genotypes (Fig. 22).

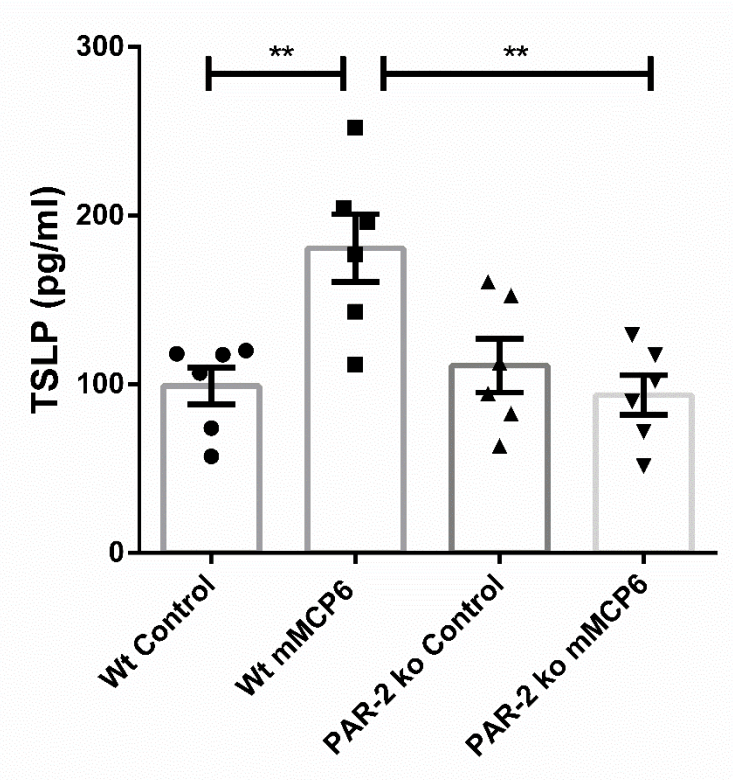


Figure 22. Exogenous mMCP6 induces TSLP in Wt skin explants, while PAR2 knockout explants are unresponsive. Skin biopsies from Wt and PAR-2 ko mice were treated with mMCP6 (10 ng/ml) for 12 h followed by measurement of TSLP levels in the supernatants using ELISA. The data is given as mean ± SEM from 6 independent experiments and analyzed using Kruskall-Wallis with Dunn's multiple comparison test (**$p<0.01$).

Taken together, the results show that MC tryptase is required for TSLP production in murine skin and most likely acting by activating endogenous PAR-2 signaling pathway.

3.3.2 PAR-2 plays an important role in the compound 48/80-mediated induction of TSLP *in vivo*

In order to test whether the *ex vivo* findings are transposable to the intact skin *in vivo*, a C48/80 (specific and potent MC degranulator) mediated MC degranulation model in Wt and PAR-2-/- genotypes was employed. The experimental scheme is shown in figure 8. C48/80 mediated TSLP abundance in the skin was significantly less in PAR-2-/- murine skin compared to their Wt equivalent (Fig. 23a).

The inhibition at mRNA expression level of TSLP was similar to protein expression of TSLP i.e. PAR-2-/- murine skin expressed significantly lower levels of TSLP transcript compared to their Wt counterpart upon C48/80 treatment (Fig. 23b).

These results suggest that C48/80 mediated TSLP response results from activation of PAR-2 signaling cascade.

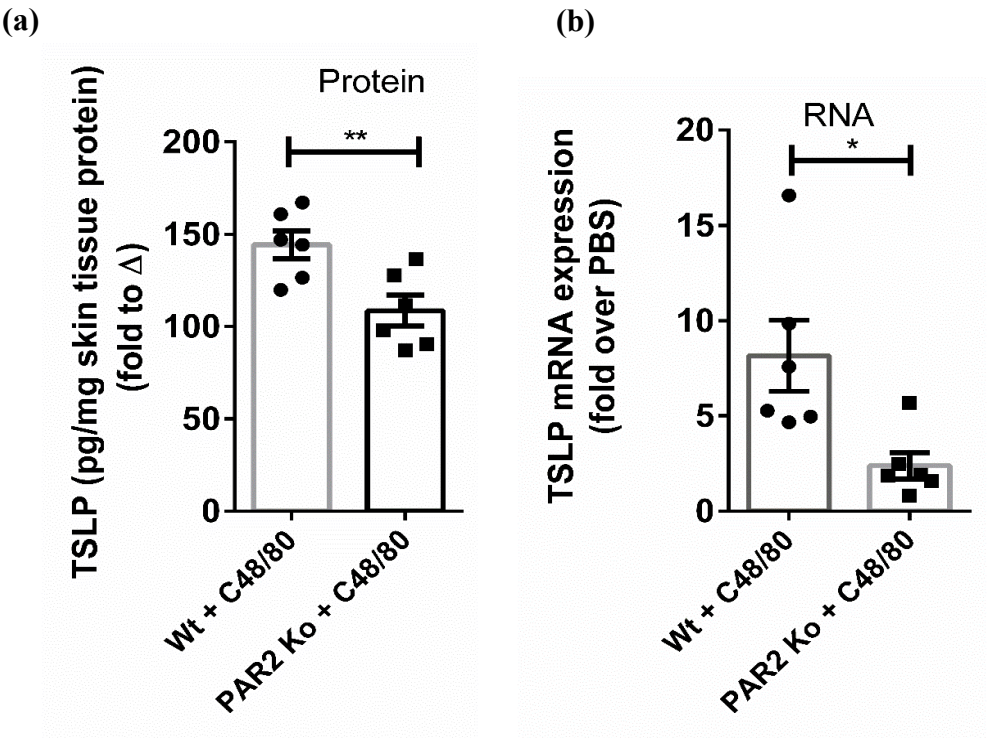
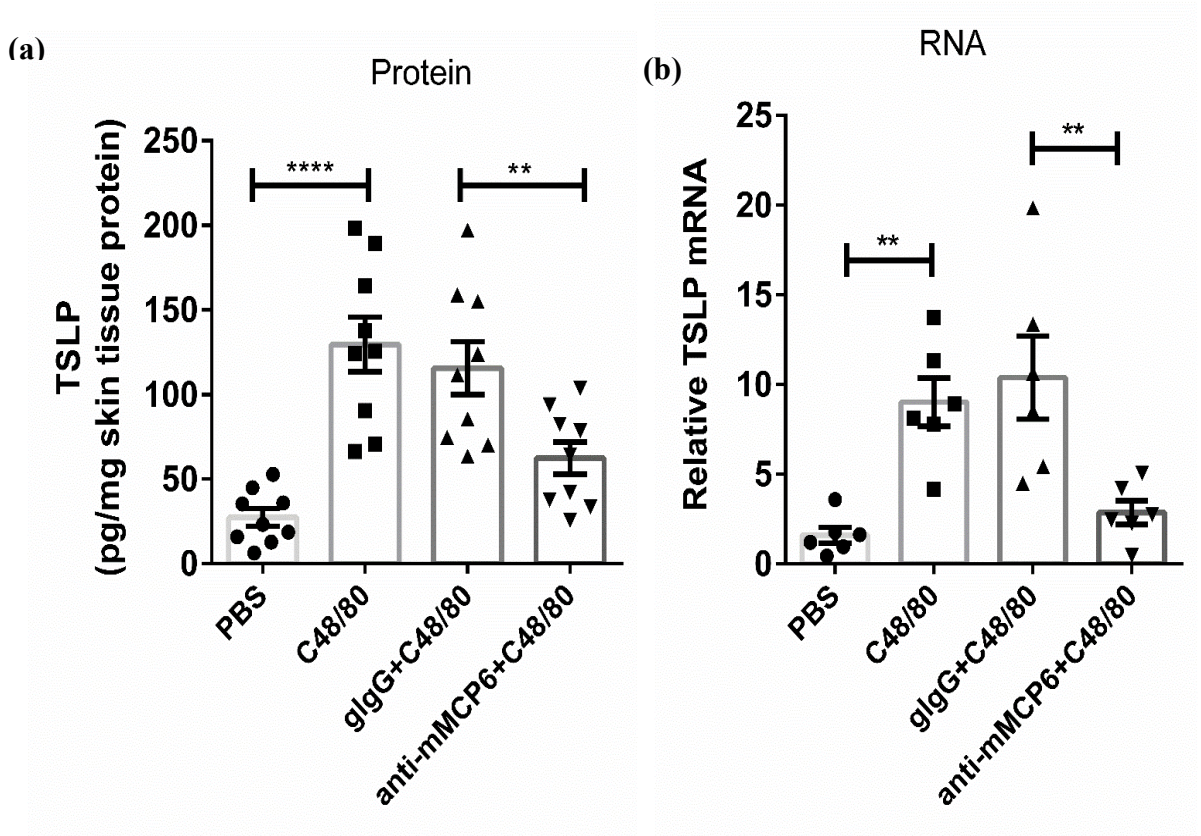


Figure 23. PAR-2 knockout mice are protected from C48/80 mediated TSLP induction *in vivo*. Each mouse was intradermally injected with 100 µg of C48/80 (PBS served as control). (a) Skin lysate was prepared and TSLP was measured using ELISA. (b) TSLP mRNA expression in murine skin. Data are shown as mean ± SEM of 6 independent experiments (*$p<0.05$, **$p<0.01$) by paired t-test and are given as fold over PBS.

3.3.3 Intradermal C48/80-triggered TSLP production in murine skin *in vivo* depends on mMCP6



To further confirm the presented ex vivo data and characterize the role of tryptase under *in vivo* conditions, a C48/80-mediated MC degranulation model after the neutralization of mMCP6 by using function-blocking antibody was employed. The experimental scheme is depicted in figure 9. As expected, intradermal C48/80 resulted in robust TSLP response compared to PBS treated group. TSLP abundance in the skin was significantly curtailed by intervention with anti-mMCP6 antibody (Fig. 24a), while interference with H1 and H4 histamine receptor antagonists showed no impact on C48/80-mediated TSLP production (Appendix Fig. 30). These results suggest that the C48/80-mediated TSLP response takes place in a tryptase dependent manner and is independent of histamine.

At the level of mRNA expression, TSLP transcripts resembled the protein data upon intradermal C48/80 treatment. In comparison to the PBS treated group C48/80 treated group resulted in profound increase in TSLP transcript levels and anti-mMCP6 antibody significantly inhibited C48/80-triggered TSLP mRNA expression (Fig. 24b). These results emphasize that C48/80-elicited TSLP induction depends on tryptase activated signaling pathway and is independent of histamine.

Figure 24. Intradermal C48/80 induces TSLP in murine skin *in vivo* – dependence on mMCP-6. Each mouse was injected intradermal with 20 µg of anti-mMCP6 or gIgG. After 14 h each mouse was intradermally injected with 100 µg of C48/80 (PBS served as control), and 8 h after mice were sacrified. (a) Skin lysate were prepared and TSLP was measured using ELISA. (b) TSLP mRNA expression in murine skin. Data are shown as mean ± SEM of 6-9 independent experiments (**p<0.01, ****p<0.0001) and was analyzed using Kruskall-Wallis with Dunn's multiple comparison test.

3.3.4 MC knockout mice are protected from C48/80 mediated TSLP induction *ex vivo*

Finally, to investigate the role of MCs in the induction of TSLP, a C48/80 mediated MC degranulation model was performed in the MC sufficient and MC knockout mice. The experimental scheme is shown in figure 9.

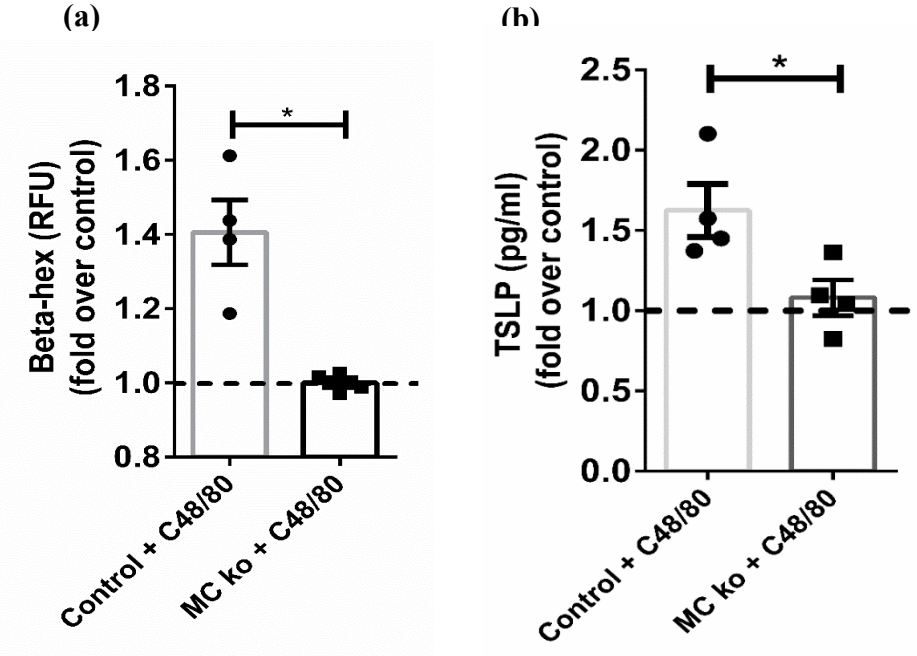


Figure 25. C48/80 mediated TSLP induction in murine skin depend on mast cells *ex vivo*. (a) Beta-Hexosaminidase and (b) TSLP was measured in culture supernatant after incubation of murine skin explants with C48/80 for 1 and 12 h respectively, using enzyme-linked immunosorbent assay (ELISA). Untreated skin explants served as control.

The data, given as mean ± SEM, from 4 independent experiments and was analyzed using unpaired t-test (*p<0.05) on the fold over control data (control set as 1)

As a positive control for MC degranulation, β-hexosaminidase was measured in the culture supernatant. C48/80-mediated β-hexosaminidase secretion was significantly less in MC-/- mice compared to MC sufficient mice (Fig. 25a), indicating strong degranulation of MCs in MC sufficient mice upon C48/80 treatment.

C48/80-triggered a robust and significantly higher TSLP response in MC sufficient mice compared to MC-/- murine skin explants (Fig. 25b).

Altogether, the presented results demonstrate that MC-/- mice are not prone to C48/80-triggered TSLP production in murine skin.

3.3.5 MC biomolecules trigger TSLP responses in human keratinocytes by tryptase *in vitro*

To examine whether the findings in the murine system are transferable in humans, primary human KCs were stimulated with 0.1% whole MC lysate after immunoprecipitation (Appendix Fig. 31) of tryptase or in presence of tryptase inhibitor nafamostat (NM) and TSLP levels were quantified in the culture supernatant. Under these experimental conditions, MC lysates induced robust TSLP production. Immunoprecipitation of MC lysate with anti-tryptase antibody significantly decreased MC lysate triggered TSLP induction (Fig. 26a). Additionally, TSLP induction mediated by MC lysate was significantly inhibited in in the presence of NM (Fig. 26b).

Collectively, MC derived biomolecules, specifically tryptase, activated pathways triggering TSLP production also in human KCs.

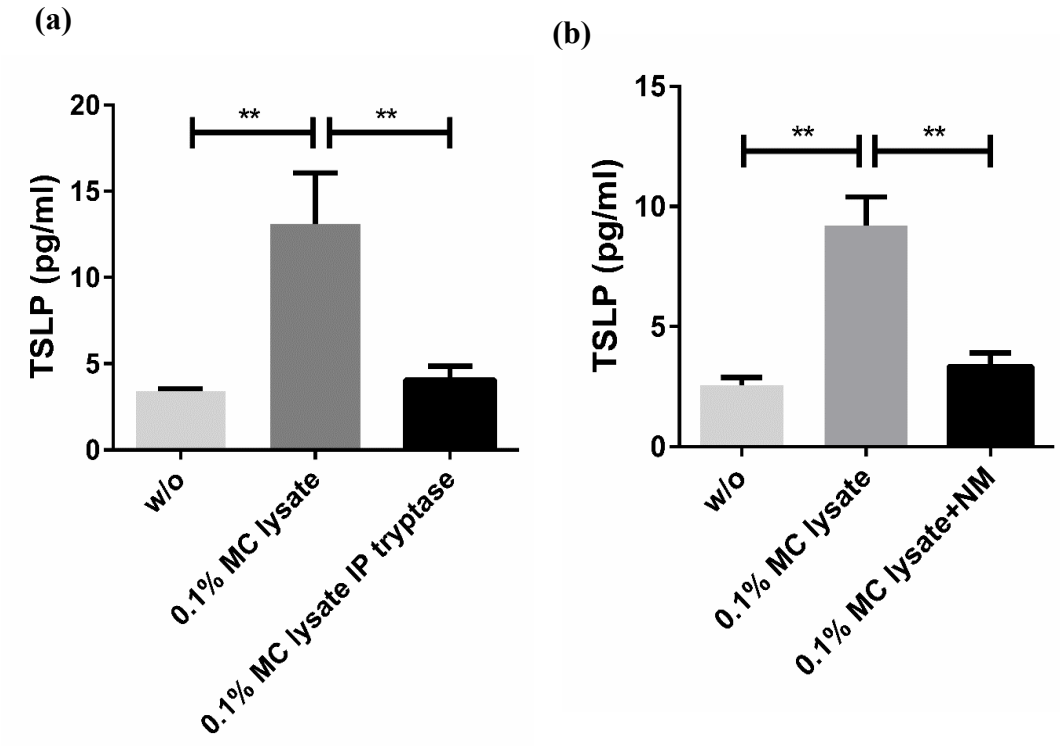
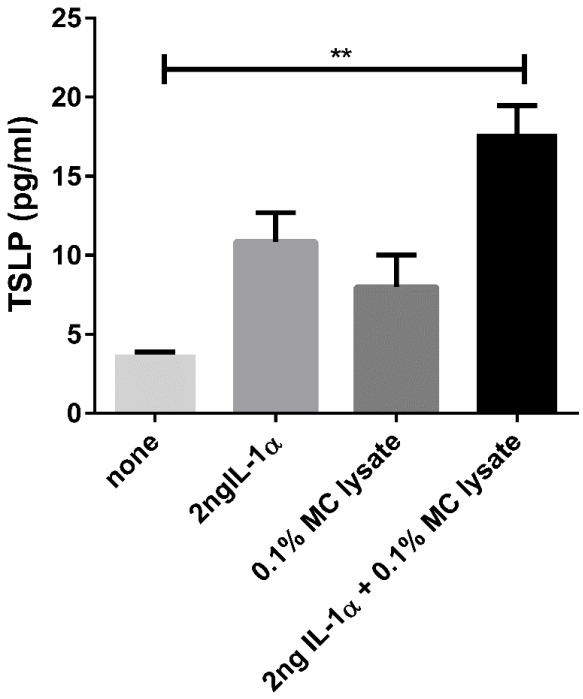


Figure 26. Products from human skin mast cells induce TSLP in human keratinocytes by tryptase dependent pathway. (a) Human keratinocytes were incubated with mast cell lysate either with/without immunoprecipitation for tryptase or (b) in presence/absence of Nafamostat mesylate. Untreated cells served as control. TSLP was measured in cell culture supernatants after 24 h using ELISA.

Data are shown as mean ± SEM of 8 independent experiments (**p<0.01) and was analyzed using Kruskall-Wallis with Dunn's multiple comparison test.

3.3.6 MC biomolecules act in concert with IL-1 to induce TSLP production by human keratinocytes

As it was seen a synergistic effect of IL-1 and PAR-2 in the co-stimulation *in vitro* assays, it was speculated that MC biomolecules may also act in concert with IL-1 to trigger TSLP



production by human KCs.

Figure 27. IL-1 and human mast cell biomolecules co-operatively induce TSLP production by human keratinocytes. Human primary keratinocytes were incubated with lysate from human mast cells or IL-1α alone or in combination. Levels of TLSP were measured in culture supernatants by ELISA after 24 h. Data are given as mean ± SEM of 4

independent experiments (**p<0.01) and were analyzed using Kruskall-Wallis with Dunn's multiple comparison test.

Given the fact that tryptase acts by activating PAR-2 pathway, it is highly likely that similar co-operative induction of TSLP upon co-stimulation with IL-1 and MC lysate will be found. To test this human primary KCs were stimulated with IL-1 and MC lysate individually as well as combined. As expected, a robust induction in TSLP production by KCs when co-stimulated with IL-1 and MC lysate compared to each individual stimuli was found (Fig. 27). These results suggest that different signaling pathways activated by respective stimuli (MC lysate mediated activation of PAR-2 and exogenous IL-1 mediated activation of IL-1 signaling cascade) act in concerted fashion to induce profound TSLP response.

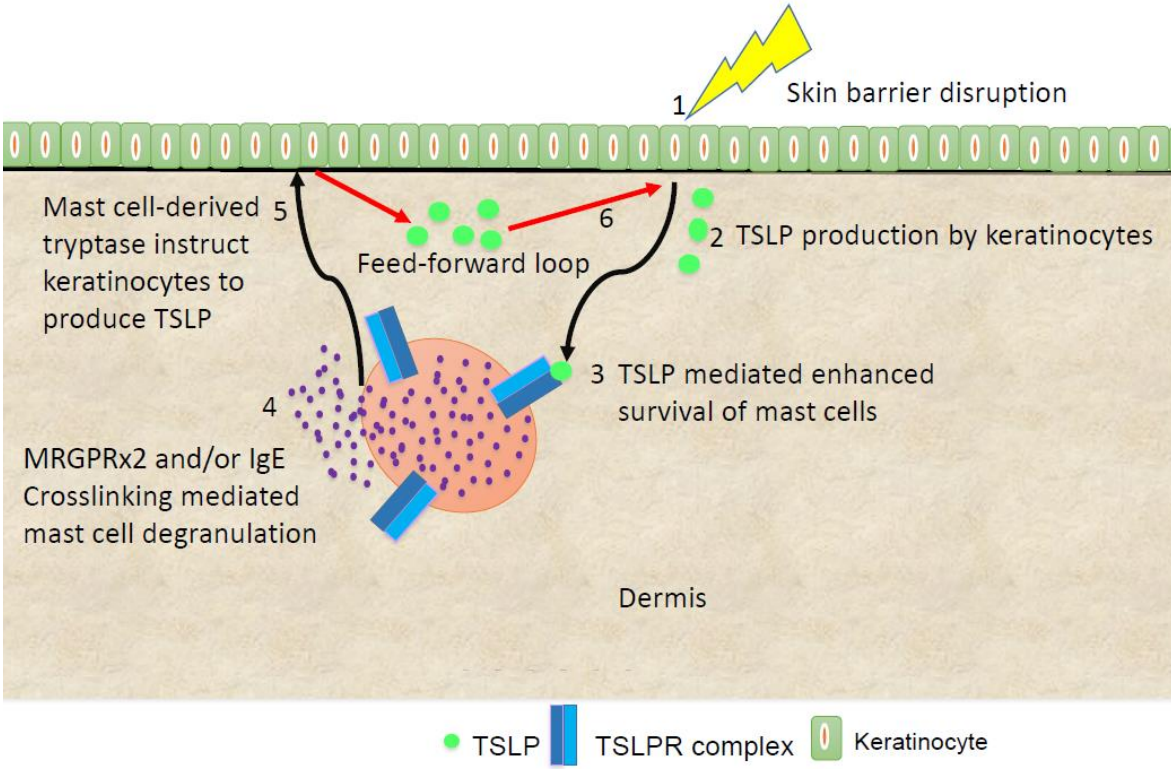


Figure 28. Graphical depiction of mast cell and keratinocyte interaction in the production of TSLP. Skin barrier disruption leads to TSLP production by KCs, which further act on the dermal MCs and enhance their survival. Upon degranulation of MCs by IgE mediated receptor crosslinking or by activation of MRGPRx2 receptor pathway, MC mediators specifically tryptase in turn act on epidermal KCs by PAR-2 mediated pathway to produce more TSLP completing the feed-forward loop.

4 DISCUSSION

TSLP is a well-known pro-inflammatory cytokine which plays a crucial role in inflammatory diseases[41,140]. It is highly upregulated in keratinocytes (KCs) upon skin barrier perturbation[36,141] and considered to be the initiator of AD. Murine skin overexpressing TSLP has been reported to develop spontaneous dermatitis alike to characteristics of human AD[34,106]. Despite the significant advances in the understanding of the role of TSLP in the development of Th2-mediated inflammation in skin and allergic diseases, the understanding of the regulatory mechanisms of endogenous TSLP production in skin inflammation and particularly in AD are still in its infancy.

In this thesis, the role of TSLP in the progression of AD and mechanisms behind skin-irritation as well as mast cells mediators mediated TSLP production was investigated. Additional aim was to better understand the role of endogenous TNF under the micro-milieu of AD and in relation to TSLPR *in vivo*.. Furthermore, in recent studies MCs were emerging as another important component of AD pathogenesis. MCs have been reported to be increased in the lesional skin of AD in case of both human as well as mice.[142] To examine the contribution of MCs in the induction of TSLP production by human KCs and in murine skin MC degranulation model *in vivo* as well as *ex vivo* was employed. Furthermore, MC lysate and purified MC mediators were analyzed in more detail *ex vivo* and *in vitro*.

4.1 TNF-/- MICE DEVELOP AGGRAVATED AD WHICH COULD BE RESCUED BY THE ABSENSE OF TSLPR EXPRESSION

AD is a complex, chronic and pruritic inflammatory skin disease. Immune dysregulation in combination with epidermal barrier defect cause development of AD.[143] Beside immunological components, pharmacological abnormalities are critical in the disease development and progression.[22]

TNF-α is a pleiotropic cytokines crucial for host defense and known for its ability to trigger inflammatory response concurrently with an immune dysregulation.[144,145] Anti-TNF therapeutics for autoimmune disorders like inflammatory bowel disease, psoriasis and rheumatoid arthritis are authorized and proving effective with rather few side-effects[146]. Most common therapies in use for disrupting the TNF function in autoimmune diseases are monoclonal antibodies, adalimumab as well as infliximab, in addition to the soluble receptor etanercept.[147,148] In the initiation and progression of AD the functional role of TNF is not

clear. Based on literature analysis, there are more indications of positive rather than negative impact of TNF on AD. Takahashi et al. (1992) reported that TNF-α was decreased in PBMCs derived from AD patients in comparison to healthy individuals.[149] In alignment, it was observed that the expression levels of TNF-α, IL-1β and IFN-γ were reduced in the skin samples of AD patients compared to skin samples obtained from psoriasis patients.[150] Furthermore, TNF-α producing dermal inflammatory dendritic cells (DCs) were reported to be lower in the skin of AD patients compared to skin of psoriasis patients.[151] The epidemiological studies from our institution as well as from different clinics provided the most noticeable evidence, which stated the manifestation of symptoms similar to AD due to side effects of anti-TNF therapy.[152-156] These observations hint towards a protective role of TNF in the framework of AD, and previously in our group we had seen that TNF deficient mice developed exaggerated AD, providing experimental evidence.[216]

TSLP is a well-known important proinflammatory cytokine in AD and asthma.[157] Increased TSLP expression has been reported in lesional skin of AD patients but not in contact dermatitis, induced by nickel.[106] TSLP is a member of IL-2 family and IL-7 type of cytokine. It can act on DCs and promote the Th2 immune response by differentiation and recruitment of Th2 cells. Naïve CD4+ cells can also be directly targeted by TSLP to induce proliferation, in response to antigen.[114] It has been shown that epithelial cell-derived TSLP can activate DCs, T cells and MCs.[130] TSLP overexpression in murine skin resulted in the development of spontaneous dermatitis.[34,106] In alignment with the literature, TSLP was significantly upregulated in AD skin of TNF-/- mice and correlated with disease severity. TNF-α is known to induce TSLP expression. Brandt *et al.* (2011) reported that human skin explants produce higher amount of TSLP, when stimulated with a combination of TNF-α, IL-1α and Th2 cytokines, but not individually.[31] Complementary findings were reported from KC cultures.[158,159] To understand why TNF-/- mice exhibit aggravated AD and weather TSLP is prime reason for AD development in these mice, a murine model was employed. To define the underlying mechanisms, an AD mouse model was performed in Wt, TNF-/- ,TSLPR-/- and TNF-/- TSLPR-/- double knockout mice to mimic the human AD scenario. The results clearly indicate that TNF-α plays a protective role against development of AD as reported in the literature. Significant worsening of the severity of AD-like lesions in the TNF-/- mice in comparison to Wt, TSLPR-/- and TNF-/-TSLPR-/- double knock out mice was observed. The analysis of the severity in the AD model was performed based on SCORAD score, a tool to evaluate clinical severity in human AD.[160] Interestingly, TSLPR-/- and TNF-/-TSLPR-/-

double knockout mice were protected from development of AD symptoms suggesting TSLP as another essential factor in the development of AD. Previously, it has been shown that MC but not the T cells were enhanced in AD lesions of TNF-/- mice and correlated with disease severity as well as to increased mRNA levels of TSLP in lesional skin.[216] It was shown by Han *et al.* that TSLP was able to induce the proliferation and differentiation of MCs as well as the number of MC was significantly higher in different organs of Wt mice in comparison to TSLP-deficient mice.[161]

4.2 SKIN IRRITATION-MEDIATED TSLP PRODUCTION DEPENDS ON IL-1 AND PAR-2 PATHWAYS

TSLP is subject to several studies and well-known as an important factor of allergic disorders in the lung, skin, and gut organs.[41,140] TSLP is abundantly expressed in KCs when the skin barrier is disrupted by genetic intervention.[36,141,162-165] Skin barrier compromised by irritation triggers TSLP response in similar manner, supporting a further connection between damaged skin integrity and TSLP expression.[20,117,121,122] Thus TSLP can be considered as initial and sensitive signal of skin barrier disruption of various sorts, making it necessary to explore the molecular mechanism of its regulation.

How skin irritation-mediated barrier disruption leads to TSLP production is an open question. Separately from limited contribution of IL-1[20], the mediators and underlying mechanisms are unclear in spite of the regular physical insults to which the skin is continually exposed in daily life and the applicability to the itch-scratch cycle to AD manifestation.

The role of IL-1 in skin inflammation is well-established. Indeed, both IL-1α and IL-1β, founding members of the family, are key coordinators to induce clinically distinct signs of skin inflammation by predominantly upregulating the expression of the entire range of inflammatory genes during sterile inflammation, skin infections and other abuses.[166-168] In response to skin injury KCs release pre-formed IL-1α[169] which in turn act as alarmin to recruit T cells to further enhance IL-1 (e.g. by inducing IL-1β expression in KCs).[170,171] IL-1α and IL-1β by acting alone or in combination with other pro-inflammatory cytokines sustain the inflammatory micro-milieu by further supporting the cytokine production by immune cells in a feed-forward loop.[170,172-174]

Beside its well-known contribution to skin inflammation, IL-1 played a role in skin irritation-mediated TSLP induction, still could support an incomplete explanation only, as

inhibition yielded in 20-50% decrease in TSLP production, relaying on the setup.[20] Thus, the question arose what alternate pathways may be activated in the irritated skin to induce TSLP production. PAR-2 turned out to be a major route, which can be activated upon barrier disruption.

Regardless of the reproduction of the cross-talk between IL-1 and PAR-2 in human and mouse, there were also discrepancies. In the mouse activation of PAR-2 alone was able to induce TSLP, and the neutralization of endogenous PAR-2 resulted in reversal of TSLP induction mediated by barrier disruption. This is of importance given the fact that efficacy of IL-1 to induce TSLP was extensively weaker in comparison to human[20], so that PAR-2 triggering on its own may adopt a major role in the mouse. However, and a key finding of this study, it was the simultaneous activation of IL-1 and PAR-2 cascades that facilitated robust induction of TSLP in both species.

The presented findings indicate that under the conditions of subclinical inflammation or mild skin irritation, as shown by minute concentrations of IL-1, could be incited measurable TSLP production when PAR-2 is concomitantly activated. The proteases to achieve this may be endogenous but also exogenous and would by themselves result in moderate TSLP induction but would be emancipated to do so in the occupancy of low levels of IL-1. In fact, various activated proteases in the skin habitat can lead to activation of *endogenous* PAR-2, including kallikreins 5 and 14.[92,175-177] In other conditions, the proteases to activate PAR-2 may be exogenic. Noteworthy, several allergens have protease activity, and presumably activate PAR-2 on KCs.[178-180] Therefore, PAR-2 is reckoned as the major sensor of proteases in the skin with numerous pathophysiological implications.[181]

Despite the importance of both signaling cascades to skin biology in health and disease, there are strangely no studies addressing their likely cooperation in the skin, and only few reports altogether. Chhabra *et al.* (2007) reported in airway smooth muscle cells, enhanced GM-CSF production by stimulation with combination of protease and IL-1 in comparison to IL-1 alone, but this apparently proceeded via a mechanism independent of PAR-2.[182] Fyfe *et al.* (2005) on the other hand, showed synergistic effect on IL-8 production by IL-1 and PAR-2, in the Caco-2 adenocarcinoma line.[183] To the best of our knowledge the last mentioned is the only report that provided evidence for cooperation between IL-1 and PAR-2.

The level at which merging of IL-1 and PAR-2 occur to increase TSLP production was demonstrated in this thesis. Noticeably, canonical NF-κB pathway can also be triggered by

PAR-2 in addition to well-known IL-1 mediated activation. It has been reported for multiples cell lines that PAR-2 triggering leads to biologically significant NF-κB pathway activation.[180,184-188]

TSLP expression majorly regulated by NF-κB and four consensus NF-κB binding sites (BSs) have been reported in its ≈4-kb-promoter region.[136,137] Stimulation with IL-1 leads to recruitment of NF-κB to two of these BSs present at -0.37 kb and -3.8 kb upstream of transcription start site, respectively.[137]

It was found that upon stimulation with IL-1 alone, NF-κB was recruited to both BSs while treatment with PAR-2-Ag alone, had no measurable impact. Interestingly the combination of PAR-2-Ag with IL-1 strongly enhanced the binding to BS4, the major site associated with activation of TSLP promoter. The same phenomena was observed even at low IL-1 concentrations.

Probably, the two functionally active NF-κB BSs differently contribute to the regulation of TSLP in an organ specific manner, e.g. between gut and skin tissue.[189] It's hypothesized that the TSLP expression at baseline and upon induction is differentially regulated by these two BSs.[190] Indeed, IL-1 (with or without PAR-2 activation) is more potent in recruiting NF-κB at BS4 in comparison to BS1, the latter rather similar to housekeeping-like functions. Increased NF-κB recruitment was reflected by enhanced activity of TSLP promoter upon co-stimulation with IL-1 and PAR-2-Ag, suggesting that binding represented the efficiency of transcriptional output.

Together, our results indicate that increased binding of NF-κB to its BSs regulate the collaboration between IL-1 and PAR-2.

The findings of this study and the sequential events are graphically depicted in figure 20.

4.3 MAST CELLS INSTRUCT KERATINOCYTES TO PRODUCE TSLP

Based on previous studies that TSLP is the initiator of AD under TNF deficiency. Moreover, enhanced number of MCs in lesional skin of TNF-/- mice and its strong correlation with the severity of AD indicates that MCs are playing a crucial role in the induction of TSLP.[216] On this background, it was presumed that MCs are upstream of TSLP in this scenario. MCs have been associated with TSLP under different conditions such as they are shown to produce TSLP themselves,[106,191] are reported to respond to TSLP,[121] (Hazzan *et al.* submitted) and can

increase TSLP production by epithelial cells.[139] Further, as shown earlier, PAR-2 plays an important role in the induction of TSLP. It has been known that MCs mediators can activate PAR-2.[192] Based on this background, the underlying mechanism behind the cross talk between MCs and KCs in TSLP production was investigated.

For this purpose, the effect of MCs supernatants/lysates and mast cell mediators was studied in the context of the induction of TSLP production. Different experimental approaches with MCs, skin explants and KCs were used for that purpose.

MCs degranulate upon stimulation with anti-IgE as a result of crosslinking of the FcεRI, which eventually leads to the release of MC mediators such as histamine and proteases.[193,194] As Total protein content of MCs consist of between 30-50% MC proteases.[195,196] We selected tryptase and histamine, as they are the two most abundant MC mediators, for their ability to induce the production of TSLP. In order to examine the role of MC mediators that may act on KCs in the skin to induce TSLP production, skin explants were incubated with the purified MC mediators or supernatants of resting MCs *ex vivo*.

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Histamine is believed to be the key factor in the communication between MCs and KCs.[69,197] Human KCs and organotypic skin models showed that histamine downregulates the expression of differentiation related proteins like *filagrin, loricrin* and Keratin in addition to desmosomal and tight junction proteins.[198] These data indicate that activation of MCs and release of histamine contribute to the initiation of AD by promoting skin barrier defects.[198] Additionally, histamine is considered as one of the prominent pruritogenic mediators. Itch is an integral part of AD, although histamine 1 receptor (H1R) antagonist was not able to ameliorate the itch in lesional skin of AD patients.[199] However it has been reported that simultaneous treatment with a H1R and histamine 4 receptor (H4R) antagonists showed anti-pruritogenic and anti-inflammatory effects, in a model of chronic allergic dermatitis in NC/Nga mice.[200]

Based on this literature and presented data, it seems clear that one of the major mast cell mediator does not appear to play a role for increased TSLP levels. Intriguingly, upon stimulation of skin biopsies with supernatants from resting MCs, TSLP levels were significantly increased. These data suggest that some of the MCs mediators other than histamine, instruct KCs to produce TSLP.

However, the supernatant obtained from unstimulated resting MCs consists of only the mediators released spontaneously (e.g. by piecemeal degranulation[201]), mediators associated with granules are at lower concentrations in the supernatants from unstimulated MCs.[202]
Interestingly, skin explants exposed to tryptase produced significantly higher amounts of TSLP in a dose dependent manner. On the other hand, histamine failed to enhance TSLP production on performing the dose response, as no single concentration of histamine resulted in enhanced TSLP production compared to untreated skin biopsies.
Therefore, tryptase was tested next as a possible elicitor of TSLP. Tryptase has been shown to induce scratching behavior by activating PAR-2[58,203] and associated with severity of AD.[204] Thus, it was investigated whether tryptase act via PAR-2 signaling cascade in skin to trigger TSLP production. PAR-2 turned out to be an essential route, which can be activated by tryptase. mMCP6 is the mouse analog of human β-tryptase, which was indeed resulted in enhanced TSLP production.

To investigate the role of PAR-2 mediated TSLP induction in the skin by tryptase, different approaches were performed. Tryptase, histamine as well as MCs supernatants/lysates were used to induce TSLP production. In one experimental model tryptase resulting from MCs supernatants and *endogenous* PAR-2 were blocked using anti-PAR-2 and anti-mMCP6 antibodies. In a second approach, mMCP6 was *exogenously* applied to activate PAR-2 pathway both in Wt and PAR-2 knockout mice. In the first scenario, we found inhibition of MCs supernatants mediated TSLP induction by anti-mMCP6 and anti-PAR-2 antibodies was found. In *exogenous* setting, induction of tryptase mediated PAR-2 dependent induction of TSLP, verifying our previous findings, was seen. To delineate whether degranulation of the MCs induce TSLP production in PAR-2 dependent manner *in vivo*, it was confirmed that C48/80 (a potent MC degranulator[57,58])-mediated degranulation of MCs induce robust TSLP production in Wt mice compared to their counterpart PAR-2 knockout mice. This points towards interaction between MCs mediators and KCs through activation of PAR-2 also under *in vivo* conditions.

Next the role of MCs mediators in the *in vivo* settings was investigated. Intradermal administration of anti-mMCP6 antibodies resulted in a significant reduction in the C48/80 induced TSLP production, in comparison to isotype control. In contrast, intervention with H1R and H4R showed no inhibition. These data confirm the *ex vivo* findings that tryptase but not histamine is the trigger factor for inducing TSLP production in the skin. Further, to

explore the role of MCs in the induction of TSLP MC deficient mice were used. Skin biopsies from control mice and MC deficient mice were incubated in presence of C48/80. As expected, MC deficient mice produced significantly less amount of TSLP in comparison to the control mice. These data suggest that MC instruct KCs to produce enhanced TSLP levels through activation of PAR-2 by tryptase.

To answer the question whether the findings in murine system apply to humans whole MCs lysates was used to stimulate human primary KCs. Interestingly, it turned out that MCs lysates were able to induce TSLP production in a tryptase dependent manner, as immunoprecipitation (IP) of tryptase lead to significant inhibition of MCs lysate-induced production of TSLP. As a complimentary approach to validate the role of MCs tryptase in inducing TSLP response nafamostat (potent tryptase inhibitor) was employed. Clearly, incubation with nafamostat resulted in significant reduction in MCs lysates mediated induction of TSLP expression. Further, in alignment with the previous findings of PAR-2 and IL-1 mediated cooperative TSLP induction, co-stimulation of KCs with MCs lysates and IL-1α resulted in robust TSLP induction. These data suggest typtase mediated activation of PAR-2 act in concert with IL-1 to induce TSLP expression by KCs.

Based on presented data it appears to be clear that tryptase is the main mediator from the MCs that results in triggering a robust TSLP production by both murine skin and human KCs. In support, it was showed by Thakurdas et al. (2007) that mMCP6-/- mice had a decreased capability to fight K. pneumonia infections, indicating an important immune protective role of mMCP6 in microbial/bacterial infections. mMCP6 was not found essential for the maturation, retention and migration of MC-committed progenitors.[205] It was reported that lesional skin of AD patients express highly upregulated tryptase like enzymes in the stratum cornneum.[206] Furthermore, elevated tryptase levels were observed in MCs from AD patients.[207] However, two studies have analyzed tryptase serum levels for suitability as a serum marker for AD, but reported no correlation between severity of AD and levels of tryptase in the serum.[208,209] In contrast, recently Sahiner *et.al.* (2018) reported serum basal tryptase levels are associated with disease severity and atopy.[204] Tryptase has been associated with AD mediated itch and scratching.[58,210] Recently, it was reported that MCs degranulation and PAR-2 activation are involved in scratching behavior and the authors reported similar increase in TSLP production in their AD model which further supports our findings.[211] Additionally, Tomoaki Ando *et al*. (2014) have reported in *Plcb3-/-* mice that MCs but not the B or T cells are crucial for the onset of spontaneous dermatitis.[212]

The recently published first body-wide expression atlas for humans (FANTOM5), revealed that MCs express highest levels of the TSLPR (CRLF2) of all cells in the body.[213,214] In agreement with receptor expression TSLP has been shown to increase MC survival.[161] (Hazzan *et al.* Submitted). Together with recent achievements in human lung MCs, which also reported functional TSLPR in these cells.[215] These findings fit the concept that MCs might be highly significant targets of the cytokine whose production they promote.

Collectively, MCs and KCs form an intricate cellular network unit in pathophysiology of TSLP by acting as regulator, producer and targets of TSLP. MCs induce TSLP by their proteases, specifically tryptase regulates TSLP production by KCs in a PAR-2 dependent manner. Furthermore, combined stimulation with MCs lysates and IL-1 resulted in enhanced TSLP response by KCs. TSLP then acts back on MCs favoring survival[161] (Hazzan *et. al*. Submitted), thereby creating a positive feed-forward loop (Fig. 28). Histamine failed to modulate the TSLP production, in contrast mMCP6 significantly induced TSLP expression by KCs in the skin biopsies, suggesting that MCs mediator tryptase is involved in instructing KCs by activating PAR-2 to produce TSLP.

4.4 CONCLUSION AND OUTLOOK

The skin is largest organ of the body and forms part of the first line of defense against pathogens or external insults. The epidermis is the outermost layer of the skin and provides the anatomical structure to form the skin barrier. Any kind of impairment to the skin barrier homeostasis triggers an inflammatory response which may lead to the development of skin diseases. In this thesis mechanisms of TSLP regulation either by barrier disruption or by MCs mediators have been investigated.

The results presented provide newer insights into the role of IL-1-PAR-2 pathways interaction in regulating TSLP in the skin. The data shown revealed that upon barrier disruption or irritant exposure keratinocytes rapidly produce robust TSLP. Moreover disturbances in skin homeostasis by mild physical irritation or upon exposure to MCs mediators resulted in induction of TSLP production. These findings indicate that TSLP in the skin acts as an alarmin, upon encounter to any kind of skin injury. The mechanistic analysis demonstrated that mild skin irritation-mediated TSLP induction results from co-activation of IL-1 and PAR-2 pathways *in vitro, ex vivo* and *in vivo*. IL-1 a well-established reciprocator

upon skin perturbation, was partially contributed in tape stripping-mediated TSLP production and PAR-2 turned out to be another important pathway involved in TSLP production. Using the ChIP and functional reporter assay it was demonstrated that the signals from IL-1-and-PAR-2 pathway converse on the TSLP promoter in human keratinocytes. To this end, skin biopsies, 3D keratinocyte cultures and co-culture of mast cells with keratinocytes will be employed. Furthermore, potential mediators and/or signaling pathways will be studied using *in vivo* mouse models. Different antibodies, agonist, antagonists and inhibitors will be used to treat the skin biopsies and cell cultures.

To better understand the importance of TSLP in absence of TNF in the development of AD, mouse model of dermatitis was performed. TNF-/- mice developed high AD severity and the severity was abolished in TSLPR-/-TNF-/- double knockout mice, similar to TSLPR-/- mice. Up to this point, mast cell number is significantly increased in the skin lesions of TNF-/- mice, indicating the involvement of mast cells in this AD model.[216] Further, analysis of these mice will include histological examination of skin, study of single cell types and factors in serum in different genotypes. To get better insight in the role of mast cells these data should be confirmed using TNF-/-MC-/- double knockout mice to prove the role of mast cells under the deficiency of TNF in AD progression.

As previous findings indicated the involvement of mast cells in the development of AD, it was asked whether mast cells can directly induce TSLP production as they have been reported to produce TSLP[191] or whether they can direct keratinocytes to TSLP during promotion of AD, as they are shown to instruct cells of epithelia to enhance TSLP production.[139] It was observed previously that bone marrow derived mast cells were not able to produce TSLP either after activation with anti-IgE alone or in combination with other cytokines.[216] Based on these findings it was hypothesized that mast cells can instruct keratinocytes to produce TSLP. To verify this hypothesis, skin biopsies were stimulated with purified mast cell mediators and resting mast cell supernatants. Tryptase as well as supernatants from resting mast cells induced significant TSLP production while histamine failed to do so. Further, to investigate the mechanism behind tryptase-mediated TSLP induction skin explants from PAR-2 knockout mice were stimulated using mMCP6. Surprisingly, only Wt murine skin explants responded to mMCP6 treatment and produced significantly high levels of TSLP, PAR-2 knockout skin explants failed to respond. These results indicated that mMCP6 induces TSLP by activating a PAR-2 pathway. Further, a

C48/80 mediated mast cell degranulation *in vivo* model was used, anti-mMCP6 in addition to H1R and H4R antagonists were employed. It was observed that anti-mMCP6 antibody inhibited C48/80 mediated TSLP induction while H1R and H4R antagonists had no effects. In the skin of PAR-2 knockout mice found significantly less TSLP expression was found compared to Wt mice upon application of C48/80.

To better understand the role of mast cells in the induction of TSLP, mast cell knockout mice were used. As expected, significantly less TSLP in the culture supernatants of mast cell knockout mice in response to C48/80 treatment was found. After observing the involvement of tryptase in the induction of TSLP with several approaches, human primary keratinocytes were investigated. Primary human keratinocytes were stimulated with lysates of mast cells, to ensure that the effects on TSLP production were from tryptase immunoprecipitation of tryptase on whole mast cell lysates was performed. Additionally, nafamostat was used to confirm the findings. In the presence of immunoprecipitated lysates or nafamostat keratinocytes did not produce enhanced TSLP in comparison to cell treated with mast cells lysates without immunoprecipitation or in the absence of nafamostat. Finally, after investigating the role of tryptase and PAR-2 in the TSLP production, it was asked if mast cell lysate can act in co-operation of low levels of IL-1, as seen before with PAR-2-Ag. To test this stimulated the human keratinocytes were stimulated with mast cells lysate individually as well as in combination with low levels of IL-1 (2 ng/ml) and found the co-stimulation resulted in highest increase in the induction of TSLP.

Inflammation and irritation of skin is an intricate system which involves the interaction of resident skin cells like keratinocytes and mast cells. Both IL-1 and PAR-2 contribute to this interaction in this scenario with a significant magnitude. Mouse skin models can be utilized to gain further insights into the mechanism of this complex network by implementing knockout mice models and by selective or collective targeting of major effector mediators in appropriate disease models.

In summary these studies reveal an important role of skin irritation and mast cells mediators in the production of TSLP and provide insights into how PAR-2 and IL-1 pathways promote early inflammatory responses in the skin.

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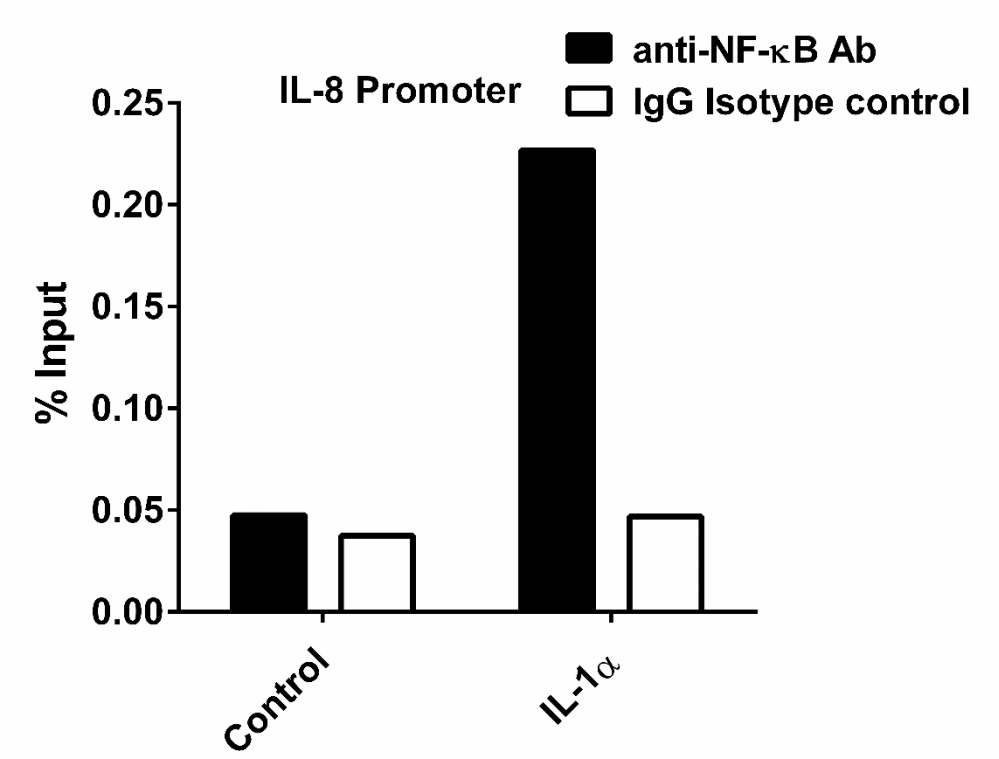
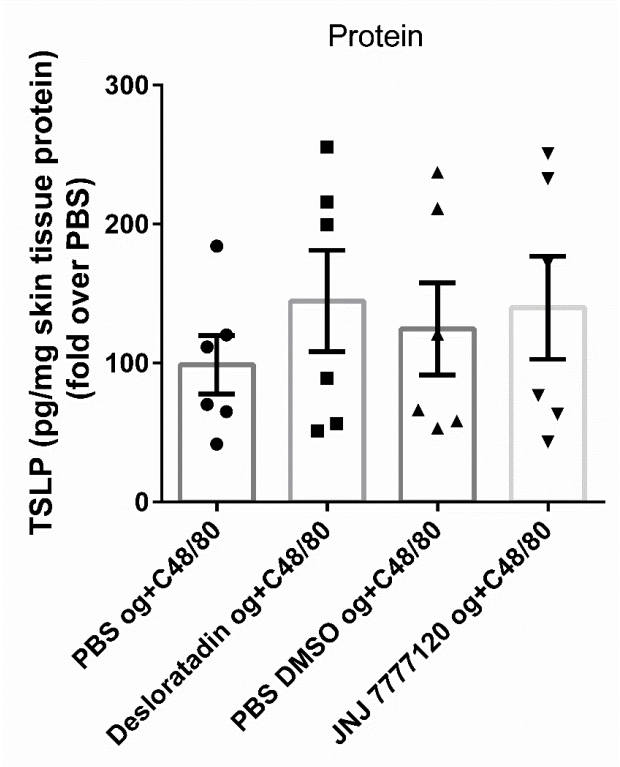
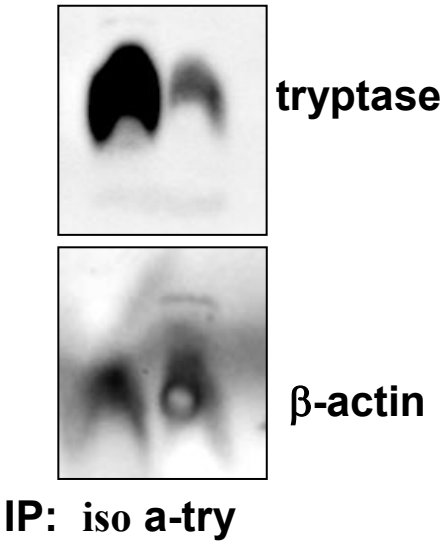
APPENDIX



Appendix Figure 29. Interleukin (IL)-1 results in the recruitment of NF-κB to the IL-8 promoter. NF-κB was recruited to the NF-κB binding site in the IL-8 promoter region in response to stimulation with IL-1α (100 ng/ml). Used as positive control for the anti-NF-κB antibody. Data representative of 3 independent experiments.



Appendix Figure 30. Intradermal C48/80 induces TSLP in murine skin *in vivo* – independent of H1 and H4 receptors. Each mouse was given oral gavage with either desloratadin (H1 receptor antagonist) or JNJ maleate (H4 receptor antagonist) or PBS. After 14 h each mouse was intradermally injected with 100 μg of C48/80 (PBS served as control), and 8 h after mice were sacrified. Skin lysatse were prepared and TSLP was measured using ELISA. Data are shown as mean ± SEM of 6 independent experiments



Appendix Figure 31. Immunoprecipitation (IP) of tryptase.

(iso = isotype, a-try = anti-tryptase)

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SELBSTÄNDIGKEITSERKLÄRUNG / DECLARATION

Hiermit versichere ich, Davender Redhu, die vorliegende Dissertation selbständig erarbeitet und verfasst zu haben. Es wurden keine weiteren Quellen und Hilfsmittel als die hier angegebenen verwendet.

I hereby declare that I, Davender Redhu, have worked and wrote this dissertation independently and did not use other than the listed support. This thesis does not exist neither in the same or similar form nor is it submitted to another examination procedure.